Characterization of the role of *TcdC* in the production of toxins A and B *in vitro* by *Clostridium difficile*

By

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A Thesis

Submitted to the Faculty of Graduate Studies,

University of Manitoba, in Partial Fulfillment

of the Requirements for the degree of

Master of Science

Department of Medical Microbiology

University of Manitoba

Winnipeg, Manitoba

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirement of the degree

MASTER OF SCIENCE

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Acknowledgements

I would like to acknowledge the mentorship of Dr. Michelle Alfa. I would also like to thank her for providing me with the opportunity and means to complete my Master's of Science degree. Her knowledge, experience and support have been crucial to my success in my program of study.

I would also like to acknowledge Drs. Michael Mulvey, John Wylie and Elizabeth Worobec for forming my graduate committee. Their time in meeting and providing feedback on my research project is much appreciated. I would like to thank them for their time and expertise reviewing this thesis.

The technical expertise and support of Nancy Olson and Patricia Degagne of the Saint-Boniface Research Centre Microbiology were invaluable in completing my research. Thank you for sharing your knowledge and experience so freely. Your friendship has made working towards this degree fun. Also thank you to David Boyd of the Nosocomial section NML for assistance with the molecular work associated with this project. I am grateful to Emma Ongsansoy of the Nosocomials section of the NML for performing PFGE analysis on *C. difficile* isolates and to the NML DNA Core facility for performing all sequencing reactions.

Thank you to the Laboratory staff at the Saint-Boniface General Hospital Clinical Microbiology Lab for your patience in providing the stool samples used in this project.

Without our parents we would be lost in this world. I would like to thank my parents, Susan and Walter Murray, for giving me direction and teaching me that with hard work I can do anything. You introduced me at a young age to the world of Microbiology and I have come to enjoy working in this field.

Finally, I would like to thank my husband, Mathieu Blanchette, who has stood beside me through this project. Even when I lost faith in myself you always believed in me. Thank you for never questioning the time and resources that went into this work. Without you I couldn't have finished, but now I'm done.

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List of Abbreviations

Abbreviation	Explanation
NAP	North American Pulsotype
cpeU	Cytopathic effect units
cfu	Colony forming units
CDAD	*C. difficile* associated disease
PMC	Pseudomembranous colitis
CPE	Cytopathic effect
CROP	C-terminal repeating oligopeptide
GEF	Guanidine exchange factor
RNAP	RNA polymerase
ATCC	American type culture collection
BA	Blood agar
FAB	Fastdious anaerobic broth
BHI	Brain heart infusion broth
CDMN	*C. difficile* moxalactam and norfloxacin agar
HFF	Human foreskin fibroblasts cells
CaCo2	Adenocarcinoma of the colon cells
TER	Tran epithelial resistance
TMH	Transmembrane hydrophobic domain
ORF	Open reading frame

Abstract

Clostridium difficile is the most common infectious cause of nosocomial antibiotic associated diarrhea. The more severe manifestations of *C. difficile* associated disease include pseudomembranous colitis, toxic megacolon and death. Two large clostridial toxins, toxin A (enterotoxin) and toxin B (cytotoxin), are responsible for the pathogenesis of *C. difficile*. A third toxin, that shares homology to the clostridial family of binary toxins, has been identified in some strains of *C. difficile*. While the role the *C. difficile* binary toxin in disease is not clear, evidence suggests that *C. difficile* strains with binary toxin cause more severe disease. While the binary toxin has been identified in all NAP1 strains of *C. difficile* the role of this toxin in the pathogenesis of CDAD has yet to be fully explained. We amplified the binary toxin *cdtB* locus from 12.5% of all *C. difficile* isolates obtained between 2000 and 2001. Binary toxin was not amplified from the clinical isolates that caused the two outbreaks that occurred during this period. Our data indicates that the presence of the binary toxin alone is not the sole virulence factor that determines whether a *C.difficile* strain will cause a nosocomial outbreak or not.

Recent outbreaks of *C. difficile* in Quebec have been linked to a hypervirulent strain of *C. difficile*. The ability of this strain to cause outbreaks has been linked to an 18 bp and 1 bp mutation in the open reading frame of the putative negative regulator of toxin production and to the presence of the binary toxin locus. The putative negative regulator of toxin production, TcdC, is expressed during early logarithmic growth. In our study we found that mutations to the *tcdC* gene were not a predictor of toxin hyper production in *C. difficile* broth culture for NAP1-related and non NAP1-related strains evaluated. Our

data demonstrated that the NAP1-related strain produced increased levels of biologically active toxin B ($4\log_{10}$ cpeU/mL) in broth culture as compared to non-NAP1 clinical isolates ($2\log_{10}$ cpeU/mL). However, the hyper toxin producing ATCC strain produced higher levels of biologically active toxin B ($5\log_{10}$ cpeU/mL) as compared to the NAP1-related strain while a non-NAP1 strain with a truncated *tcdC* gene did not show increased toxin production in broth culture.

By evaluating stool from CDAD patients within 4 hours of disease diagnosis, we demonstrated a correlation between toxin B in the stools of patients with CDAD and toxin produced by corresponding clinical isolates in broth cultures.

A novel finding from our research was that there was an increase in the efficiency of sporulation, as measured by initiation and rate of spore conversion in broth culture for the NAP1-related strain when compared to a non-NAP1 strain. The combination of increased sporulation efficiency and increased toxin production in the NAP1-related strains may result in an increase in spread and pathogenesis in this strain.

In summary our data provide invaluable insight into the role of the TcdC protein in hyperproduction of toxin by the NAP1 strain and a novel aspect we demonstrated was the efficient sporulation ability of this strain. The data from our study furthers our understanding of the virulence of this unique strain of *C.difficile*.

Introduction

1. General overview of *C. difficile* associated disease

Clostridium difficile is a large gram positive, spore forming, anaerobic bacillus belonging to the family Clostridiaceae. *C. difficile* is the causative agent of *C. difficile* associated disease (CDAD). The symptoms of CDAD range from mild to moderate diarrhea to severe pseudomembranous colitis (PMC) (Voth and Ballard 2005).

Antibiotic-associated disease

Antibiotics have had an enormous impact on infectious disease over the past century. The availability of penicillin offered physicians the first effective treatment for many once debilitating or even fatal bacterial infections. The start of the antimicrobial era was not without drawbacks. Many of the antibiotics had serious side effects; including drug toxicity, hypersensitivity reactions and antibiotic associated disease. Antibiotic-associated disease, with symptoms ranging from mild diarrhea to severe pseudomembranous colitis (PMC), proved to be a life-threatening side-effect of the new antimicrobial drugs. The first description of PMC, as a case of pseudomembranous enterocolitis, occurred in 1893 as a post operative complication of a gastrostomy (Finney 1893). Following this first description more sporadic incidences of PMC were described as a complication of gastrointestinal injury. After the introduction of penicillin, PMC became more common and was almost exclusively linked to prior exposure to penicillin. Other clinical diagnosis, including; intestinal obstruction; ischemic cardiovascular disease; uremia, heavy metal poisoning (Bartlett 1980), proton pump inhibitors and gastric acid suppressors (Poutanen and Simor 2004) have been linked to development of PMC lesions. Any condition of surgical intervention that disrupts the colonic microflora

has the potential to increase the risk of antibiotic-associated disease, however, the administration of antibiotics remains the main risk factor for PMC (Poutanen and Simor 2004). PMC lesions are characterized by the formation of exudative plaques along the intestinal tract. Upon histological examination PMC lesions are composed of neutrophils, fibrin, mucin and cellular debris (Figure 1) (Bartlett 1979; Poutanen and Simor 2004).

C. difficile, the etiologic agent of antibiotic associated disease

Soon after the advent of penicillin many investigators observed that administration of penicillin had fatal effects to small laboratory animals such as hamsters and guinea pigs. The lethality of the antibiotics to small laboratory animals was also observed for new antibiotics. In effect, antimicrobial therapy was more harmful to the animals than the infections being treated. All animals showed deleterious effects following antibiotic exposure including hair loss and an unsteady gait before succumbing to antibiotic induced death (Bartlett, Onderdonk et al. 1977; Bartlett, Chang et al. 1978). Upon autopsy of laboratory animals a hemorrhagic cecitis, the hamster model of CDAD, was observed. Although many mechanisms were proposed to explain this phenomenon, initial investigations into the etiologic agent of hemorrhagic cecitis in hamsters focused on the identification of an infectious organism that was responsible for disease. This was based on observations that germ free mice were able to tolerate antibiotic therapy without any adverse effects. It was believed that this animal model could provide information relating to the recent increase in cases of antibiotic associated disease in humans (Bartlett, Onderdonk et al. 1977; Bartlett, Chang et al. 1978). In the 1950s, research to identify the

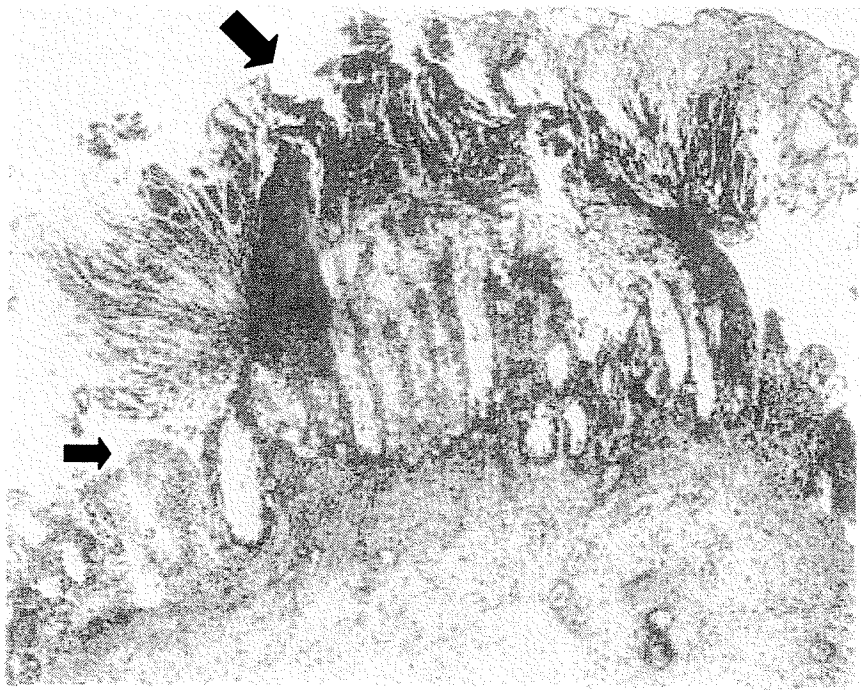


Figure 1. Histological section of colonic mucosal gland consistant with PMC

Pseudomembranous lesion consisting of fibrinous cap (large arrow) with surrounding inflamed mucosa (small arrow) and cellular debris from a human colonic mucosal gland. Haematoxylin and eosin X 35 (adapted from Shortland, Spencer et al. 1983).

etiologic agent of PMC focused on *Staphylococcus aureus*. Examination of stools on autopsy demonstrated *S. aureus* in the stools and pseudomembranes of patients on antibiotic therapy (identification by culture and gram stain). *S. aureus* was also known to produce an enterotoxin. However, early investigators were unable to reproduce the hemorrhagic cecitis observed in small laboratory animals using isolates of *S. aureus* or its enterotoxin. Also, *S. aureus* levels were elevated in all patients on antibiotic therapy and not all patients developed antibiotic-associated disease (Young 1965; Bartlett, Onderdonk et al. 1977).

In the 1970s a renewed interest in antibiotic-associated disease led investigators to search for another microbial agent that could be the cause of PMC. Development of PMC in patients strongly correlated to clindamycin use (Bartlett, Onderdonk et al. 1977). A preparation of the cecal contents of hamsters following challenge with clindamycin produced a cytopathic effect (CPE) on WI-38 cells. Further studies demonstrated that the CPE was neutralized by pre-incubation of stool preparations with gas gangrene antitoxin. As such, a cytotoxin produced by the bacterial genus, *Clostridium*, was associated with the development of PMC. Gas gangrene antitoxin contains antibodies to factors from the five clostridial strains known to cause gangrenous lesions. Isolation and testing of the components of the gas gangrene antitoxin identified the *C. sordellii* antitoxin as the neutralizing antitoxin in the gas gangrene mix. As no evidence of *C. sordellii* was found in stools and the toxin produced by *C. sordellii* was unable to produce hemorrhagic cecitis in hamsters, it was determined that a cross-reactive toxin from another *Clostridium sp.* caused disease. *Clostridium difficile* was the only *Clostridium sp.*

isolated from hamsters following challenge with clindamycin that produced toxin that caused a CPE similar to the effect seen in cecal contents. The CPE was neutralized by *C. sordellii* antitoxin. Infection of hamsters with *C. difficile* cultures and the cytotoxin purified from the supernatants were able to reproduce hemorrhagic cecitis in hamsters (Bartlett, Onderdonk et al. 1977; Bartlett, Chang et al. 1978). Thus, the requirements of Koch's postulates for the identification of an etiologic agent of hemorrhagic cecitis, the hamster model of antibiotic-associated disease, were fulfilled. To demonstrate that the hamster model could be extended to humans a diagnostic test was designed to identify a cytotoxin in stools of humans with antibiotic-associated disease. A stool was positive for *C. difficile* toxin if preparations of the stool gave the characteristic CPE on a fibroblast cell line and if the CPE could be neutralized by pre-incubation with either *C. sordellii* or gas gangrene antitoxin. *C. difficile* was also isolated from the stools and cultured in broth media. Culture supernatants were tested for CPE in the same manner as for the stools. Approximately 99% of stools and cultures from patients with antibiotic-associated disease were positive for the toxinogenic *C. difficile* (Bartlett, Onderdonk et al. 1977; Bartlett, Chang et al. 1978). This provided the necessary evidence demonstrating that a cytotoxin produced by *C. difficile* is the etiologic agent of antibiotic-associated disease in humans.

C. difficile-associated disease

C. difficile is a large gram positive bacillus that forms sub-terminal endospores resistant to heat and alcohol. Hall and O'Toole first isolated and characterized *C. difficile* from the stools of neonates in 1935. *C. difficile* was initially identified as the anaerobic

pathogen *Bacillus difficile* owing to the difficulty encountered when trying to isolate and culture this micro-organism from stools (Voth and Ballard 2005). In the late 1970s *C. difficile* was determined to be the etiologic agent of antibiotic-associated disease (Bartlett, Chang et al. 1978). Since then studies have shown that *C. difficile* is responsible for the entire range of disease observed in antibiotic associated disease. *C. difficile*-associated disease (CDAD) encompasses the spectrum of disorders ranging from mild/moderate diarrhea to PMC, toxic megacolon, colonic perforation and death (Poutanen and Simor 2004). *C. difficile* survives on environmental surfaces as a heat resistant endospore. Although the importance of different transmission vectors is unclear it is assumed that the endospores are transferred on items contaminated with fecal matter. When antibiotics are administered the ecology of the colonic microflora is altered. If *C. difficile* spores are present in the colon the disruption to the microflora usually due to antibiotics leads to the germination of the spores. Vegetative *C. difficile* proliferates with the incumbent production of two toxins, toxin A (enterotoxin) and toxin B (cytotoxin), that act as the primary virulence factors of *C. difficile*. Toxin A and toxin B are ADP-glucosyltransferases that transfer the glucose moiety of ADP-glucose to the Rho superfamily of GTPases, blocking the signaling cascade induced by these proteins. A third toxin belonging to the family of clostridial binary toxins has also been identified. Binary toxin acts as an ADP-ribosyltransferase of G-actin and blocks actin polymerization. However, the exact role of binary toxin in CDAD has not been clearly defined.

Epidemiology of CDAD

CDAD is a multi-hit disease. Two criteria are required for the development of CDAD; i. disruption of the gut microflora; ii. presence of toxigenic *C. difficile* that proliferate and produce toxins. The major risk factor associated with acquiring CDAD is previous exposure to broad spectrum antibiotics. Antibiotics linked to an increased risk of CDAD have evolved with prescribing practices. Historically, increased risk of CDAD has been linked to tetracycline and chloramphenicol (1950s), clindamycin and ampicillin (1970s) and more recently fluoroquinolones (Bartlett 1979; Loo, Poirier et al. 2005). All antibiotics except vancomycin, erythromycin and some aminoglycosides have been linked to cases of CDAD. Aside from antibiotics, chemotherapeutic agents have been linked to an increased risk of developing CDAD. Currently, antibiotic exposure remains the main risk factor for the development of PMC, however, any disruption to the colonic microenvironment, including gastrointestinal surgery, chemotherapeutic agents and gastric acid suppressors, increases the risk of developing PMC (Poutanen and Simor 2004).

C. difficile is primarily a nosocomial pathogen, however recently CDAD has emerged in the community. Although reports of incidence vary, *C. difficile* has been identified as a part of the normal gut flora in ~10% of healthy adults and up to 50% of healthy neonates. In the mid-1970s PMC was reported in 10% of patients treated with clindamycin (Tedesco, Barton et al. 1974). CDAD is now considered the most common cause of PMC and is responsible for 20-30% of cases of antibiotic-associated diarrhea (Hyland, Ofner-Agostini et al. 2001). The national prevalence rates of nosocomial CDAD in a

1997 Canadian surveillance project were reported as 13.0% of all diarrheal stools submitted (95% CI 9.5% to 16.5%). The mean number of nosocomial CDAD was 66.3 cases/100,000 patient days (95% CI 37.5-95.1) and 5.9 cases/1,000 patient admissions (95% CI 3.4-8.4). These numbers are in accordance with the incidence of nosocomial CDAD reported in other countries (Hyland, Ofner-Agostini et al. 2001). In 2002 in Manitoba the number of cases of lab confirmed CDAD (936) exceeded the number of lab confirmed cases for all other enteric bacterial pathogens combined (503). Routine enteric pathogens included *Salmonella sp.*, *Shigella sp.*, *Campylobacter sp.*, *Yersinia spp.* and *E. coli* O157:H7 (data provided at the 2002 *C. difficile* Symposium for the Manitoba Infection Control group).

Current state of disease

In the early 2000s the epidemiology of CDAD started to evolve. An outbreak of *C. difficile* in Montreal, Quebec resulted in disease characterized by an increase in incidence, severity of disease and attributable mortality when compared with past CDAD epidemiology data for the region. The predominant clone circulating during the Montreal outbreak (84% of strains isolated) has emerged in recent years as a hypervirulent strain of *C. difficile*. This strain has been typed as the North American PFGE type-1 (NAP1), restriction endonuclease type BI, PCR ribotype 027 and toxinotype III (Loo, Poirier et al. 2005). For the remainder of this thesis the epidemic strain will be referred to as the NAP1 strain. The NAP1 strain produced 16 and 23 times the amount (in μg/L) of toxin A and toxin B, respectively, when compared to toxinotype 0 strains. Toxinotyping is a currently described method of typing *C. difficile* strains based on restriction patterns of

the PaLoc region. The wild-type PaLoc was described as Toxinotype 0 (Rupnik, Avesani et al. 1998). The increase in toxin production in the NAP1 strain has been attributed to deletions in the *tcdC* open reading frame on the pathogenicity locus (Spigaglia and Mastrantonio 2002; Warny, Pepin et al. 2005; MacCannell, Louie et al. 2006; Curry, Marsh et al. 2007). The NAP1 strain has since been identified in seven Canadian provinces, the United States, the United Kingdom, the Netherlands, Belgium and France (MacCannell, Louie et al. 2006; Tachon, Cattoen et al. 2006; van den Hof, van der Kooi et al. 2006; Curry, Marsh et al. 2007).

To illustrate the impact of the emergence of the NAP1 strain consider the epidemiology data from Sherbrooke, Quebec, a city 140 km southwest of Montreal. Incidence of CDAD increased 10-fold from 1991-2003 rising to 866.5/100,000 inhabitants. Mortality rates (within 30 days of diagnosis) of CDAD in 2003 were 13.8%, with a 6.9% attributable mortality rate, as compared to 4.7% in 1991/1992 (Pepin, Valiquette et al. 2004). From 1991-2002 rates of metronidazole treatment failure remained stable at 9.6% of 688 patients. During the 2002-2003 Quebec outbreak, metronidazole treatment failed in 25.7% of 435 patients (Pepin, Alary et al. 2005). Morbidity, defined as a case requiring colectomy or ICU admission as a result of CDAD had also increased in 2002/2003 as compared to the previous ten years.

2. Factors contributing to *C. difficile* pathogenesis

The pathogenicity locus

The two major *C. difficile* virulence factor genes, *tcdA* and *tcdB*, are carried on a 19 kb region of the *C. difficile* genome called the Pathogenicity Locus (PaLoc) with three additional ORFs (*tcdR*, *tcdE* and *tcdC*) that code for regulatory factors (Figure 2) (Hundsberger, Braun et al. 1997; Voth and Ballard 2005). The PaLoc has been identified in all strains of *C. difficile* that have been associated with human disease. Although not an independent mobile element the PaLoc is a distinct genetic element with well defined borders. Integration of the PaLoc into the *C. difficile* genome is conserved in all strains. In non-toxinogenic strains of *C. difficile* the 19 kb PaLoc is replaced by a 115 kb region (Braun, Hundsberger et al. 1996).

Toxin A and toxin B are both glucosyltransferases that glucosylate the Rho superfamily of GTPases at threonine 35/37. Toxin A is a more potent enterotoxin than toxin B and as such it is believed to play an important role in disrupting the enteric epithelial layer inducing enteric symptoms and allowing toxin B access to the underlying cell layers. Toxin B is a more potent cytotoxin and as such plays a putative role in the systemic manifestations of CDAD (Poutanen and Simor 2004). However, the emergence of toxin A-negative/toxin B-positive strains of *C. difficile* capable of causing disease indicate that toxin B may play a more important role in early disease than originally proposed (al-Barrak, Embil et al. 1999; Alfa, Kabani et al. 2000). There is no currently available explanation for the differences in cell specificity observed for toxin A and toxin B. The C-terminal repeating oligopeptide (CROP) region of toxin A has been shown to bind to

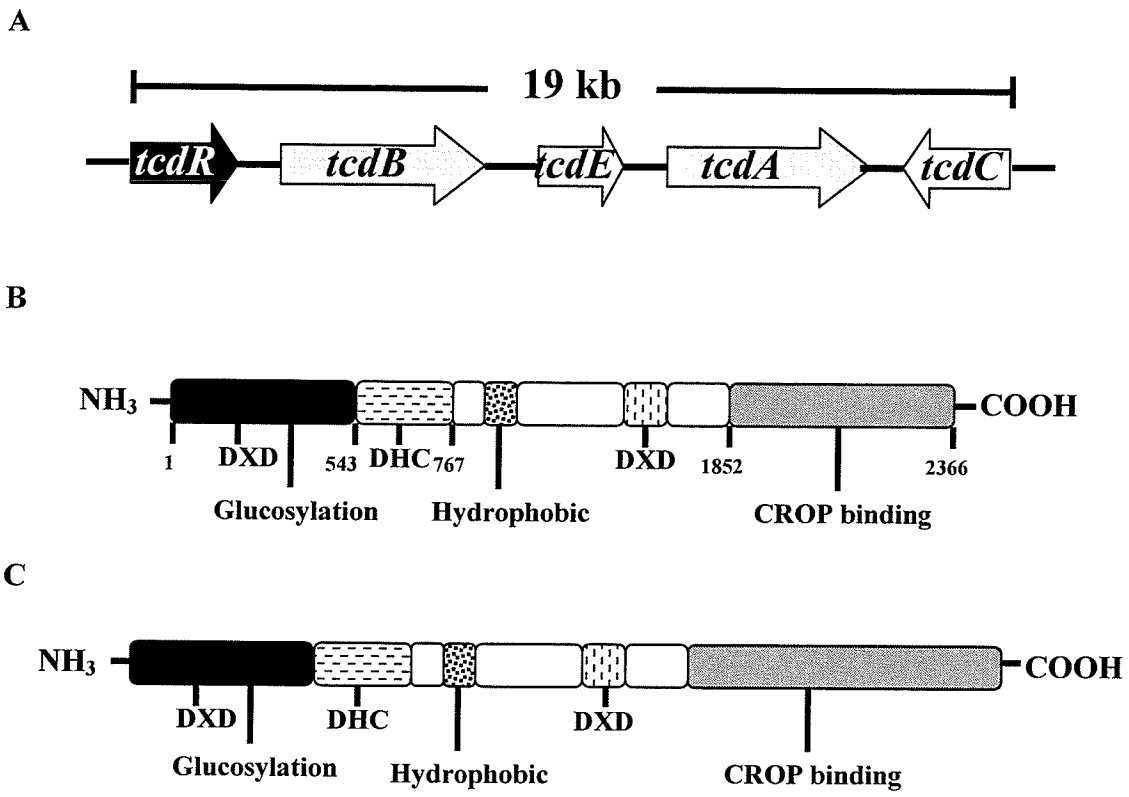


Figure 2. Genetic arrangement of the PaLoc and functional arrangement of toxin A and toxin B

The *C. difficile* pathogenicity locus (A) is composed of five open reading frames; the two toxins, *tcdA* (enterotoxin) and *tcdB* (cytotoxin); *tcdR* (alternative sigma factor); *tcdE* (putative holin protein) and *tcdC* (putative negative regulator). The functional arrangements of toxin B (B) and toxin A (C) are shown below the PaLoc (adapted from Aktories 2007).

the trisaccharide Galα1-3Galβ1-4GlcNAc. However, it is doubtful whether this trisaccharide is present on human colonic cells as there is no functional α-galactosyltransferase in the intestine. As such colonic cells cannot form α-galactosyl bonds (Jank, Giesemann et al. 2007). The cell surface receptor recognized by toxin B has not been identified (Jank, Giesemann et al. 2007). The identification of these receptors will provide further insight into the specificity of toxin A and toxin B.

Toxin A and toxin B bind to receptors on the cell surface via their CROP regions (Figure 2). The toxins are taken up by the cells via receptor mediated endocytosis. Acidification of the early endosome results in the insertion of the hydrophobic transmembrane domain in the endosomal membrane, transporting the N-terminal glucosyltransferase domain to the cytosol. The N-terminal domain is cleaved from the full length protein via inositol phosphate mediated auto-cleavage at amino acid 543 (Reineke, Tenzer et al. 2007). A cysteine protease domain (amino acids 543-767) is necessary for auto-cleavage of toxin A and toxin B. Residues 537 (aspartate), 653 (histidine) and 698 (cysteine) form the catalytic triad of the cysteine protease (Egerer, Giesemann et al. 2007) (Figure 2). The N-terminal glucosyltransferase domain interacts with Rho GTPases in the cytosol transferring the glucose moiety of ADP-glucose to threonine 37 (*Rho*A) or threonine 35 (*Cdc*42, *Rac*) (Figure 3). GTPases act as a switch, turning signaling cascades on (GTP bound) or off (GDP bound). A cycle whereby guanidine exchange factors (GEF) catalyzes the replacement of GDP for GTP. An intrinsic phosphorylase activity hydrolyses the γ-phosphate of the GTP to GDP, returning the GTPase to its inactive state. The threonine 35/37 glucosylation sites are highly conserved in Rho GTPases and are

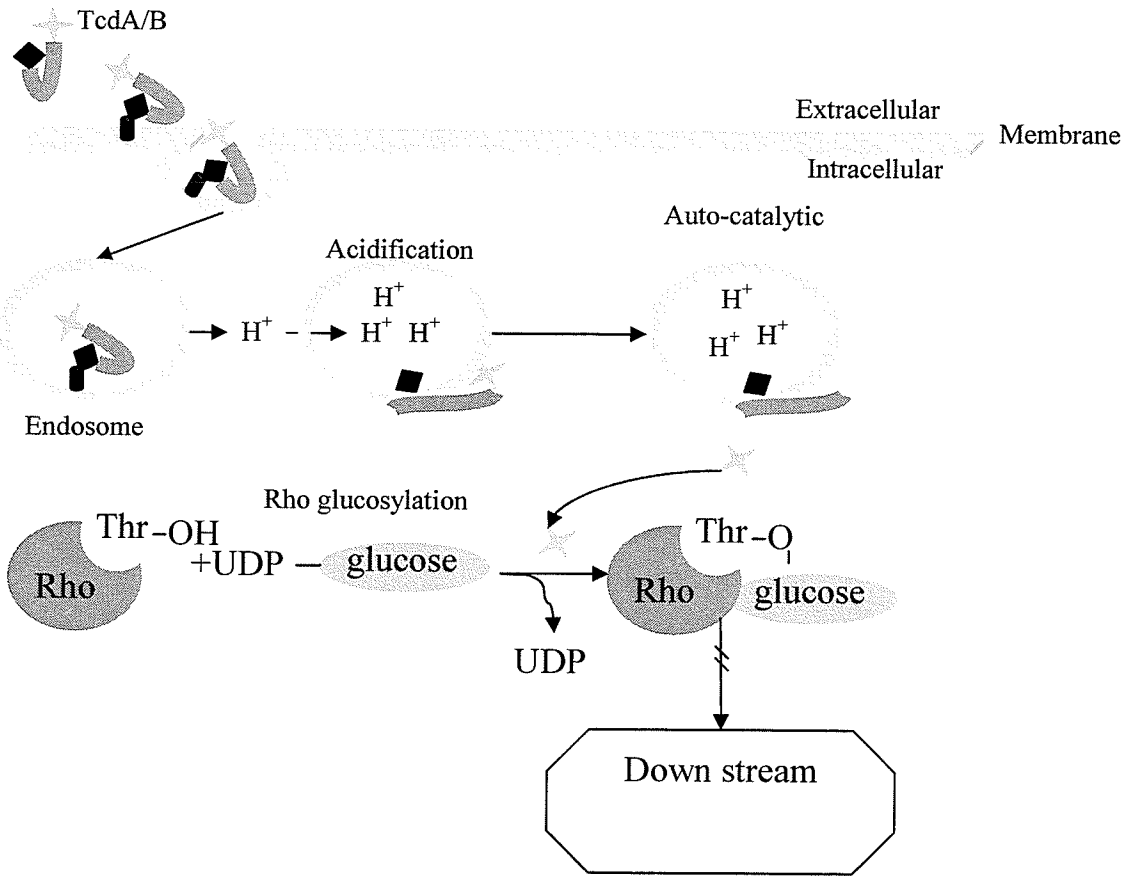


Figure 3. Mechanism of toxin A and toxin B uptake by eukaryotic cells

Toxin A/B are taken into the cell by receptor mediated endocytosis. Toxin A binds the Galα1-3Galβ1-4GlcNAc trisaccharide. No cell surface receptor has been identified for toxin B. Acidification of the endosome leads to a conformational change in toxin A/B and the transmembrane domain inserts into the endosomal membrane. The auto-catalytic cleavage domain cuts the protein releasing the N-terminal catalytic domain. The catalytic domain catalyses the glucosylation of Thr37 (*Rho*A) or Thr35 (*Cdc*42, *Rac*) inhibiting the effect of the Rho GTPases. Downstream effects of Rho inhibited include epithelial barrier functions, migration, production of reactive oxygen species, cytokine production, Immune cell signaling, wound repair and phagocytosis (Adapted from Aktories 2007).

essential to nucleotide binding. Glucosylation of this site blocks the exchange of GDP for GTP, inhibiting the activation of the GTPase. Downstream effects inhibited by the disruption of Rho GTPase signaling cascade are responsible for the pathology observed in CDAD (Jank, Giesemann et al. 2007). Cellular processes affected by blocking the GTPase signal cascade include; deregulation of actin cytoskeleton polymerization; disruption of epithelial barrier functions; cell migration; phagocytosis; cytokine production; wound repair; immune cell signaling and production of reactive oxygen species (Aktories 2007; Jank, Giesemann et al. 2007). Interestingly, RhoB has been found to be up regulated in toxin A and toxin B damaged cells. RhoB in these cells is resistant to inhibition by glucosylation and remains in a hyperactive state. This is one possible explanation for the overactive inflammatory response observed in some *C. difficile* infections (Gerhard, Tatge et al. 2005; Jank, Giesemann et al. 2007). In brief, toxin A and toxin B block the exchange of GTP for GDP in the Rho superfamily of GTPases causing the pathology observed in CDAD.

Regulation of toxin production and secretion is regulated by the products of the three accessory genes on the PaLoc, *tcdR*, *tcdE* and *tc*dC. Early transcriptional analysis of The PaLoc showed that the first four ORFs (*tcdR*, tcdB, *tcdE* and *tcdB*) are transcribed as monocistronic and bicistronic transcripts in the same orientation as *tcdA* and *tcdB* early in stationary phase. However, the *tcdC* ORF was transcribed in the opposite direction during the exponential growth phase (Figure 4). As such researchers proposed a putative model of gene transcription where the *tcdR* and *tcdE* gene products, TcdR and TcdE, were involved in the up-regulation of *tcdA* and *tcdB* transcription and secretion of mature

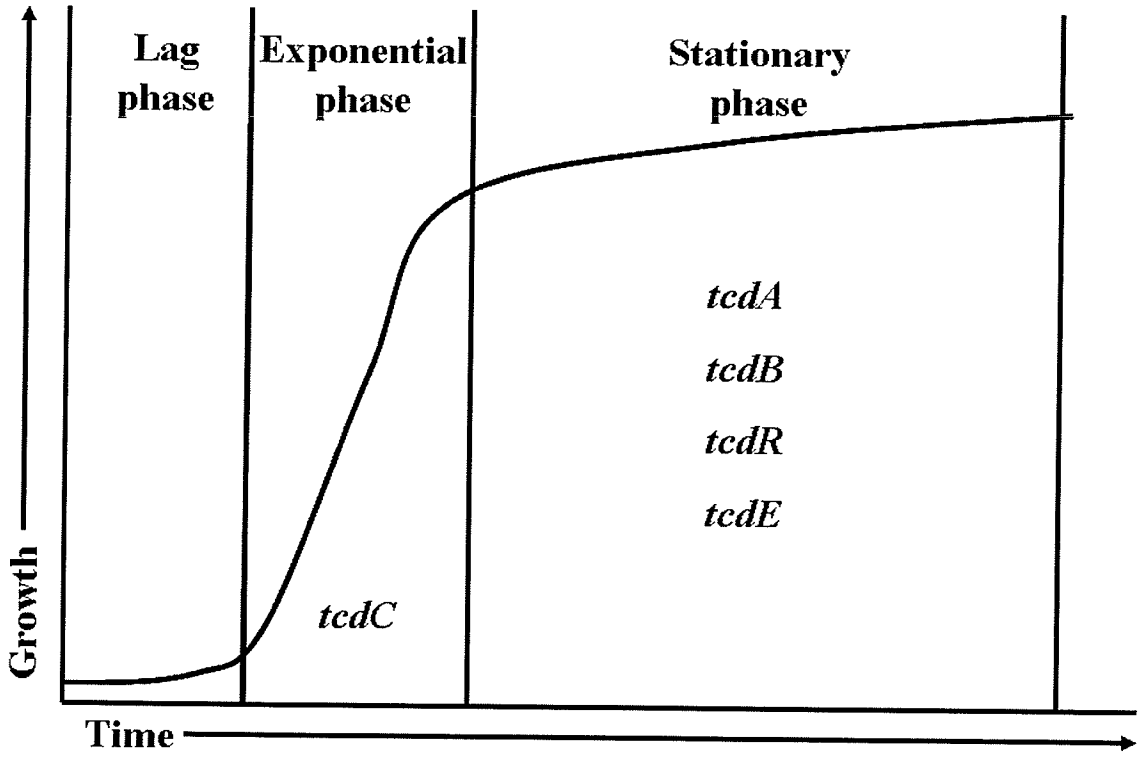


Figure 4. Gene transcription during *C. difficile* growth curve

The genes on the pathogenicity locus are expressed during the different phases of the growth curve. The phases of the growth curve are indicated above the curve and the gene products synthesized are indicated below the curve.

toxins, respectively. The *tcdC* gene product, TcdC, was identified as a putative negative regulator of toxin A and toxin B expression (Hammond, Lyerly et al. 1997; Hundsberger, Braun et al. 1997). TcdR has been identified as an alternative sigma factor belonging to group 5 of the σ^{70} family. The TcdR protein interacts with RNA polymerase (RNAP) to form the RNAP-TcdR holoenzyme. The RNAP-TcdR holoenzyme initiated transcription from the *tcdA* and *tcdB* promoters (P*tcdA* and P*tcdB*) at a significantly greater rate than the RNAP alone in P*tcdA*/P*tcdB-gusA* reporter fusion assays (Mani and Dupuy 2001). The RNAP-TcdR holoenzyme specifically initiates transcription from P*tcdA* and P*tcdB* as the RNAP-TcdR holoenzyme is unable to bind to and initiate transcription from the *C. difficile* glutamate dehydrogenase promoter (P*gdh*). Toxin A and toxin B expression was inhibited *in vivo* in glucose or amino acid rich media, suggesting that toxin A and toxin B are expressed in response to stress to the *C. difficile* bacteria (Dupuy and Sonenshein 1998). However, the mechanism of catabolite repression of TcdR in *C. difficile* has not been described.

The *tcdC* gene is located downstream of the *tcdA* gene on the PaLoc. The protein product of *tcdC* is a 231 amino acid protein. TcdC was originally determined to be a putative negative regulator of *tcdA* and *tcdB* expression. This assumption was based on the initial transcriptional analyses of the PaLoc. The *tcdC* gene is expressed during early exponential phase in the opposite direction to the other genes on the PaLoc (Hundsberger, Braun et al. 1997). Western blot analysis of membrane and cytosolic fractions of *C. difficile* using anti-TcdC antibodies were used to determine the subcellular localization of TcdC. TcdC was found in Triton-X soluble and insoluble membrane fractions and not

the cytoplasmic fractions demonstrating that TcdC is localized in the cytoplasmic membrane (Govind, Vediyappan et al. 2006). A recent study revealed that TcdC interacts with the RNAP-TcdR holoenzyme to block the formation of the open complex with P*tcdA* and P*tcdB* inhibiting transcription from these promoters. These results indicate that the inhibition of the RNAP-TcdR holoenzyme by TcdC would result in the down regulation of *tcdA* and *tcdB* without acting directly on the toxin genes being affected (Matamouros, England et al. 2007). This is contrary to the previously proposed mechanism of TcdC where it was assumed that TcdC would act directly on the *tcdA* and *tcdB* gene promoters.

Two deletions to the *tcdC* gene have been identified in the NAP1 strain. The first, an in-frame 18 bp deletion in the oligonucleotide repeat region results in a 6 amino loss in the TcdC protein (231 to 225 amino acids). This mutation was suggested to be the cause of the increased toxin production observed in the NAP1 strain (Warny, Pepin et al. 2005). Purified TcdC with the 18 bp deletion (225 amino acid protein) inhibited the formation of the RNAP-TcdR holoenzyme and blocked transcription from P*tcdA/B* in the *gusA* reporter fusion assay (Matamouros, England et al. 2007). In all NAP1 strains a second single base pair deletion (Δ117) upstream of the 18 bp deletion has been identified that results in a frame shift that leads to an early stop codon resulting in a 61 amino acid truncated protein. A TcdC construct missing the 88 N-terminal amino acids ($TcdC_{89-232}$) was able to reduce transcription from P*tcdA* and P*tcdB* in the *gusA* reporter assay (Matamouros, England et al. 2007). This indicates that the protein binding domain on TcdC that interacts with the RNAP-TcdR holoenzyme is not located in the conserved

amino acids of the truncated protein. This frame shift mutation is a more likely explanation than the 18 bp deletion for the hyper-production of toxin A and toxin B in the NAP1 strain.

The *tcdE* ORF is a 501bp sequence located between the *tcdB* and *tcdA* ORFs on the PaLoc. Initial sequence analysis of *tcdE* indicated that *tcdE* shows homology to bacteriophage holin proteins. Bacteriophage holins are cytolytic toxins released by bacteriophages during lytic infections, initiating release of progeny bacteriophage. Expression of the *tcdE* gene in *Escherichia coli* caused lysis of the bacteria following insertion of TcdE into the bacterial membrane and pore formation (Tan, Wee et al. 2001). Homologues of the *tcdE* gene have been identified in three bacteriophages, ΦC2, ΦC6 and ΦC8 (Goh, Chang et al. 2005). The proposed function of TcdE in *C. difficile* is to punch holes in the bacterial cell wall allowing the release of toxin A and toxin B (Tan, Wee et al. 2001; Goh, Chang et al. 2005; Voth and Ballard 2005).

Binary toxin

A clostridial binary toxin has been identified in some strains of *C. difficile* (Popoff, Rubin et al. 1988). The binary toxin locus is carried on a ~4 kb region of the *C. difficile* chromosome and contains two ORFs that code for the binding (*cdtB*) and enzymatic (*cdtA*) sub-units of the binary toxin. The enzymatic sub-unit acts as an ADP-ribosyltransferase that shares homology with members of the iota family of clostridial binary toxins. The putative mechanism for the uptake and action of the *C. difficile* binary toxin are described in (Figure 5). The importance of binary toxin in the pathogenesis of

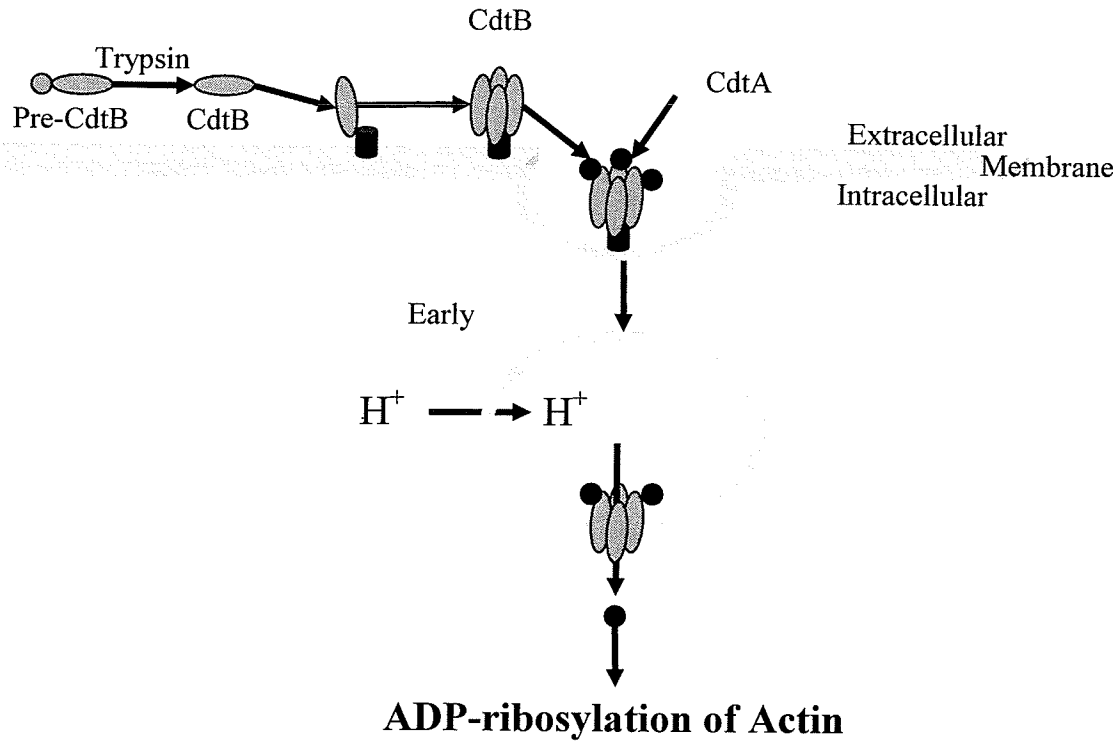


Figure 5. Putative mechanism for the uptake of *C. difficile* binary toxin in eukaryotic cells

The putative mechanism of binary toxin has been described based on the mechanisms of other members of the iota family of clostridial binary toxins. Briefly, a precursor to the binding component (pre-CdtB) is released to the extracellular matrix where it undergoes activation by proteolytic cleavage of the leader region. Activated CdtB binds to receptors on the cell surface (unknown) and forms a heptameric complex. The enzymatic component (CdtA) binds to the heptameric CdtB and is taken into the cell via receptor-mediated endocytosis. In the acidified early endosome the CdtB complex under goes a conformational change, creating a pore in the endosomal membrane allowing CdtA into the cytosol. CdtA is an ADP-ribosyltransferase that ribosylates G-actin inhibiting polymerization and extension of F-actin (Adapted from Voth 2005).

C. difficile has yet to be elucidated although strains possessing the binary toxin locus are thought to be more virulent than strains without binary toxin. Studies have been unable to detect a cytopathic effect using different cell culture assays (Popoff, Rubin et al. 1988; Gulke, Pfeifer et al. 2001). Infection of hamsters with *C. difficile* strains negative for toxin A and toxin B but positive for binary toxin caused mild gastrointestinal symptoms but was not lethal. In the rabbit ileal loop assay binary toxin was found to cause fluid accumulation (Geric, Carman et al. 2006).

Sporulation

C. difficile is able to survive in the environment by forming heat resistant sub-terminal endospores (Arcuri, Wiedmann et al. 2000). These spores are not killed by most common cleaning agents used as hospital cleaners. As such sporulation plays an important role in the survival and spread of *C. difficile* in the nosocomial environment. One of the most effective means of killing *C. difficile* spores on environmental sufaces is chlorine bleach. Other strong oxidizing agents have demonstrated some efficiency for killing *C. difficile* spores however use of bleach and other strong oxidizing agents limits the lifespan of environmental surfaces and equipment (Perez, Springthorpe et al. 2005).

Another challenge to infection control posed by *C. difficile* spores is the spread of spores on the hands of healthcare workers. As *C. difficile* spores are highly resistant to killing by alcohol, the widespread use of alcohol-based hand hygiene solutions have been questioned for patients with CDAD. The alcohol-based hand hygiene solutions are effective at killing vegetative *C. diffiicile*, however, the spores are not killed by alcohol.

The most effective means of removing *C. difficile* spores from the hands of health-care workers remains thorough hand-washing in warm, soapy water. In an era where incidence and severity of CDAD are increasing the use of alcohol-based hand washes alone cannot replace traditional hand-washing (Anonymous 2007).

A model for sporulation has been described in *Bacillus subtilis*. Two mechanisms of regulation necessary to the efficient sporulation have been described in depth for *B. subtilis*. The *Spo*0 phosphorelay terminates in the activation of *Spo*0A using a mechanism similar to that observed in two component cell signaling pathways. *Spo*0A is transcriptional activator/repressor that acts on the 0A promoter box (5'TGNCGAA3') in the *Spo* operon (Hoch 1993; Arcuri, Wiedmann et al. 2000; Sebaihia, Wren et al. 2006). A second mechanism for the regulation of sporulation in *B. subtilis* is the activation of a sigma factor cascade, activating the transcription of sporulation genes (Arcuri, Wiedmann et al. 2000). The signals initiating sporulation in *B. subtilis* have yet to be described. All the sporulation related genes identified in the *B. subtilis* model have been identified in all members of the *Bacillus* and *Clostridium* species including *C. difficile* suggesting a conserved mechanism of sporulation in these genera (Arcuri, Wiedmann et al. 2000; Sebaihia, Wren et al. 2006). However studies have not been able to show the role of the phosphorelay and the sigma factor cascade as a mechanism for control of sporulation in *Clostridium sp.* Unlike in *B. subtilis* where sporulation is triggered by nutrient deficiency, in *Clostridium sp.* sporulation is triggered in the presence of low levels of oxygen and environments where there are rich carbon and nitrogen sources (Sauer, Santangelo et al. 1995).

Early sporulation is characterized by the formation of a septum dividing the cell into the pre-spore and mother cell compartments. An RNAP sigma factor, σ^K (*sigK*), localizes to the cytoplasm of the mother cell and initiates the expression of σ^E. Transcription of the early sporulation genes, involved in production of the cortex of the spore, in the mother cell is initiated by σ^E. In *B. subtilis* the σ^K gene is interrupted by a 48 kb prophage-like *sigK* intervening sequence (skin). Initial studies focus on the excision of *skin* as a means of differentially regulating the σ^K gene. However, studies in *B. subtilis* mutants lacking the *skin* sequence showed no sporulation defects and this mechanism of regulation was proven to be unnecessary for efficient sporulation of *B. subtilis*. In *C. difficile* a similar 14.6 kb prophage-like insertion in the *C. difficile* $sigK^{Cd}$ ($skin^{Cd}$) was described. $Skin^{Cd}$ inserts into the $sigK^{Cd}$ gene by site specific recombination. An ORF directly upstream of the 3' end of $sigK^{Cd}$ and in the 14.6 kb $skin^{Cd}$ codes for a putative resolvase/invertase enzyme. This resolvase/invertase putatively mediates the insertion and removal of $skin^{Cd}$ from $sigK^{Cd}$. Unlike in *B. subtilis* the $skin^{Cd-}$ mutants of *C. difficile* were not efficient spore formers. The mechanism of sporulation proposed involves the regulated excision of $skin^{Cd}$ which allows the initiation of *sigK* transcription. In $skin^{Cd-}$ mutants constitutive transcription of *sigK* creates a repressor feed-back loop, inhibiting sporulation and producing the sporulation deficient phenotype observed (Haraldsen and Sonenshein 2003).

The impact of antibiotics on CDAD

C. difficile is the primary etiologic agent of antibiotic associated disease, being responsible for 15-20% of antibiotic induced diarrhea and the primary cause of PMC. Antibiotics play a multifaceted role in CDAD. The disruption of the intestinal microflora, allowing the germination of *C. difficile* spores colonizing the intestine is a necessary condition for the establishment of CDAD. Although the triggers for toxin production and secretion in *C. difficile* are unknown some antibiotics have been linked to increased toxin production using *in vitro* models (Freeman, O'Neill et al. 2003; Baines, Freeman et al. 2005; Baines, Saxton et al. 2006; Freeman, Baines et al. 2007). Treatment with vancomycin or metronidazole remain as the preferred first-line treatments for CDAD.

The "Antibiotic Bowel" was initially described as the disruption of the gut microflora, including the reduction of coliform bacteria and concomitant increase in the bacteria inhibited by the coliform bacteria. Bacteria isolated from early cases of "Antibiotic Bowel" included *Staphylococcus*, *Proteus* and *Pseudomonas* (Young 1965). *In vitro* gut models of CDAD have demonstrated that antibiotics, predominantly clindamycin, third-generation cephalosporins and aminopenicillins, impacting the balance of anaerobic bacteria in these models increased the growth and toxin production of *C. difficile* strains (Baines, Saxton et al. 2006). The current *in vitro* gut model consists of three reaction vessels set-up in series to mimic the different segments of the intestinal tract (i.e. pH control). The first reaction vessel is seeded with emulsified fecal matter from healthy volunteers (confirmed *C. difficile* culture negative) and broth media is used to feed this

vessel at a rate mimicking the transit time of fecal matter through the intestine. The pH in the first reactor vessel mimics that of the stomach, the second vessel mimics the the small bowel, the third vessel the large bowel. The reactor vessel series is allowed to stabilize for two weeks before inoculation with *C. difficile* spore (~4$\log_{10}$cfu/mL). The levels of fecal bacteria, *C. difficile* vegetative bacteria and spores, toxin B titres as well as antibiotics can be determined in time course experiments in the gut model (Freeman, O'Neill et al. 2003). The use of this model has shown that maintenance of the gut microflora is sufficient to inhibit the germination of *C. difficile* spores (Baines, Freeman et al. 2005). This is consistent with the clinical observation that piperacillin/tazobactam, an antibiotic with limited activity on the gut microflora, is rarely associated with CDAD.

Despite initial indications to the contrary, fluoroquinolones have emerged as being associated with an increased risk-factor for development of CDAD (Loo, Poirier et al. 2005). This is primarily due to the emergence of the fluoroquinolone resistant NAP1 epidemic strain. An increase in use of fluoroquinolones in Quebec hospitals paralleled the emergence of the NAP1 strain in the province (Gerding 2004; Loo, Poirier et al. 2005; Biller, Shank et al. 2007). Elimination of competing flora allowed the fluoroquinolone resistant *C. difficile* to survive and spread in the nosocomial environment.

Interruption of the aggravating antibiotic is often sufficient to resolve cases of mild antibiotic associated diarrhea. In cases of PMC, however, it is necessary to treat CDAD with courses of either oral metronidazole or oral vancomycin. Studies comparing the

efficacy of metronidazole and vancomycin show that vancomycin is slightly more effective at treating CDAD than metronidazole (Pepin, Valiquette et al. 2004; Pepin, Routhier et al. 2006). The rate of relapse is also greater with metronidazole than with vancomycin. Despite the marginally better outcome seen with vancomycin metronidazole remains the first-line antibiotic used to treat CDAD with vancomycin reserved to treat multiple relapses of CDAD. This is done to try and limit the emergence of vancomycin resistant *Enterococci* in the nosocomial setting (Gerding 2000; Johnson, Sanchez et al. 2000; Pepin, Alary et al. 2005). When vancomycin and metronidazole therapy fails to resolve PMC, then colectomy or ileoectomy to remove the infected sections of the bowel becomes necessary. Various alternatives to metronidazole/vancomycin therapy are being investigated as potential treatments for CDAD. One of the alternative therapies is the re-population of the intestinal flora using probiotic micro-organisms. Among the micro-organisms being investigated, *Lactobacillus rhammnose GG* and *Saccharomyces boulardii* are the most studied. However, the data that exists to support the effectiveness of treatment of CDAD with probiotic bacteria is mainly anecdotal based on retrospective case studies rather than double-blinded, case controlled studies of efficacy (Hickson, D'Souza et al. 2007; Karpa 2007; Segarra-Newnham 2007). The exploitation of the recently developed *in vitro* human gut model (Freeman, O'Neill et al. 2003) to explore the effect of dosing with probiotic bacteria on the germination of *C. difficile* spores and proliferation of toxins should provide valuable information into the potential of probiotics in the treatment of CDAD. The potential of anionic exchange resins (cholestyramine, tolevomer, cholestipol) to bind toxin A and toxin B in the cecal content of patients with CDAD has

been investigated. However, despite early promising outcomes following treatment with anionic exchange resins, the success of this treatment remains sporadic and is only indicated for cases of CDAD refractory to more conventional forms of treatment. The use of immunoglobulin against toxin A has been used to treat CDAD in incidences of metronidazole and vancomycin failure however no data exists to support the use of toxin An immunoglobulin (Gerding 2000). A vaccine to toxin A/toxin B is currently under construction but there is no current vaccine to prevent CDAD.

3. Knowledge gaps in *C. difficile* pathogenesis

Molecular basis of toxin regulation

Much progress has been made in the past decade to elucidate the roles of the various PaLoc gene products in the regulated synthesis and secretion of toxin A and toxin B. However, many gaps in the understanding of the underlying triggers and mechanisms of these proteins remain (Voth and Ballard 2005). The most important gap in knowledge is the role of the TcdC protein in inhibiting transcription from *tcdA* and *tcdB* during the exponential growth phase. Recent experimental data demonstrates that TcdC over expressed in P*tcdA/B-gusA* (*C. perfringens*) reporter fusion assays inhibits transcription from these promoters by blocking the formation of the RNAP-TcdR holoenzyme (Matamouros, England et al. 2007). As these experiments were performed in *C. perfringens* as opposed to *C. difficile* it is not clear if these protein interactions would be conserved in the *C. difficile* genetic background. Also, it was necessary to over-express TcdC in this model to achieve the inhibition of the RNAP-TcdR holoenzyme (Matamouros, England et al. 2007). More work needs to be done to determine if TcdC

can inhibit the holoenzyme at physiological concentration, both in *in vitro* culture of *C. difficile*, as well as in animal models of CDAD. There is increasing urgency attached to clarifying the role of TcdC in toxin A and toxin B transcription as a truncated form of TcdC in the NAP1 strain has been attributed to the hypertoxin production seen in these strains (Warny, Pepin et al. 2005; MacCannell, Louie et al. 2006; Curry, Marsh et al. 2007). Not all researchers are convinced that the TcdC truncation is the only factor influencing the toxin hyperproduction observed in these strains (Freeman, Baines et al. 2007). Experiments using the *in vitro* gut model have demonstrated that while toxin production is seen sooner for the NAP1 strain when compared to the UK epidemic strains using this model toxin, production still corresponds to the beginning of stationary phase which is reached earlier for the NAP1 strain (Freeman, Baines et al. 2007).

Studies on the TcdE protein have yet to demonstrate a definitive role for this protein in *C. difficile*. The homology of TcdE to certain bacteriophage holin proteins had led researchers to assign a putative role in the secretion of toxin A and toxin B from *C. difficile*. Bacteriophage holin proteins are produced during lytic bacteriophage infections. The holins are pore forming proteins that migrate to the bacterial cell wall where they insert into the membrane and induce cell lysis and release of the bacteriophage. TcdE expressed in an *E. coli* vector will form pores in the bacterial cell wall inducing cell lysis (Tan, Wee et al. 2001). These experiments, performed in the gram negative *E. coli*, have not been repeated in the gram positive *C. difficile* or in a gram positive model bacterium. As such, it is not possible to conclusively describe TcdE as a pore forming protein involved in toxin secretion in *C. difficile*.

Importance of sporulation in pathogenesis

Like most members of the *Clostridium* and *Bacillus* genera of bacteria, *C. difficile* is able to form heat resistant spores to survive in adverse conditions. It is widely accepted that these spores are important to the survival of *C. difficile* on environmental surfaces, providing a vector for transmission in the nosocomial setting. Despite the accepted role of *C. difficile* spores in transmission of disease very little is known in regards to the triggers stimulating sporulation or if the spores play a role in pathogenesis. In most *Clostridium sp.* sporulation is a response to trace amounts of oxygen in the presence of a surplus of carbon and nitrogen sources (Sauer, Santangelo et al. 1995). Despite this the specific trigger of sporulation in *C. difficile* is unknown. Studies have found that an inverse relationship exists between toxin production and sporulation in *C. difficile* (Akerlund, Svenungsson et al. 2006; Freeman, Baines et al. 2007). This would suggest that sporulation does not play a direct role in host tissue damage, however, there is no conclusive evidence that can eliminate a role for spores in pathogenesis.

Toxin and spore levels in stools of patients with CDAD

Many experiments studying the kinetics of toxin production and sporulation have been performed in *in vitro* and animal models of CDAD. However, there is very limited data on the levels of toxins and of spores in guts of patients with active CDAD. In one study higher fecal toxin levels were correlated with increased frequency of diarrhea ($P<0.0001$) in 20 patients with CDAD (Akerlund, Svenungsson et al. 2006). In this study stools were collected and frozen before being tested. In previous studies freezing of the toxins

reduced the biological activity in cell culture (Freeman and Wilcox 2003). However, in the Akerlund et al. study toxin levels were analyzed by a quantitative Ridascreen assay. Whether the toxin levels would be affected by prior freezing in this assay is unknown. Information available on toxin levels produced by *C. difficile* strains from *in vitro* culture data with toxin levels measured in μg/L amounts has demonstrated that the NAP1 strain produces 16 and 23 fold more toxin A and toxin B, respectively, when compared to toxinotype 0 clinical isolates (Warny, Pepin et al. 2005). As this method fails to take into account differences in the biological activity of toxin A and toxin B proteins it provides limited useful information on this strain.

Patients with CDAD are thought to shed spores in the stools of patients with CDAD. Studies on spore levels in the stool have attempted to enumerate spores in the stool by spore staining (Akerlund, Svenungsson et al. 2006). This is not a sensitive measure of spore levels in stool. Although *C. difficile* spores are metabolically inactive and cannot produce toxins, intuitively there may be a molecular trigger that shuts down toxin synthesis and initiates sporulation. In order to further elucidate the role of spores and sporulator triggers, more information on spore levels in stool of patients with CDAD would be useful.

4. Hypothesis and objectives

The mutations to the putative negative regulator of toxin production, TcdC, and production of binary toxin by the NAP1 strain of *C. difficile* are the primary virulence

factors implicated in the increased pathogenicity of this strain. The hypotheses of this thesis are that;

Increased pathogenesis of CDAD in strains of* C. difficile *that produce higher levels of biologically active toxin A and toxin B is not due to mutations in the putative negative regulator* tcdC *and, as such, the detection of mutations in the* tcdC *does not indicate increased pathogenesis. Efficient sporulation in hypertoxin producing* C. difficile *strains contributes to the ability of the strain to cause an epidemic. The presence of binary toxin, which has been associated with the* tcdC *mutations, does not correlate with the ability to cause outbreaks.

The first objective of this research was to assess the biological activity of toxin A and toxin B at different times in the *C. difficile* growth curve for strains with differing *tcdC* alleles. The biological activity was assessed by the Caco2 tight-junction (toxin A) and cytotoxin (toxin B) assays (Mahida, Makh et al. 1996; Alfa, Kabani et al. 2000; Alfa, Swan et al. 2002). Genetic characterization of the Pathogenicity locus (PaLoc) will be performed to identify possible variations in the PaLoc that could impact toxin production. Secondly, the frequency of previously described *tcdC* mutations and binary toxin in isolates obtained during an outbreak of *C. difficile* will be determined. An important aspect of *C. difficile* associated disease which has not yet been described is the quantification of biologically active toxin A, toxin B and the level of spores in stools of patients diagnosed with CDAD. The final objective of this thesis is to determine the quantity of biologically active toxin A and toxin B as well as spores in the stool of patients with CDAD.

Materials and Methods

1. Bacterial strains

Clinical isolates of *C. difficile* for the analysis of the PaLoc were obtained from Dr. Paul Levett of the Saskatchewan Provincial Health Laboratory (*C. difficile* strains 57A and 83) and from the National Microbiology Laboratory (NML) culture collection (*C. difficile* strains 1083, 79A292 and 81A330). *C. difficile* control strains, ATCC 43255 and ATCC 43594, were obtained from the American Type Culture Collection (ATCC). They are representative of high and low toxin producers, respectively.

The diagnosis of CDAD was confirmed if the stool sample was positive for *C. difficile* glutamate dehydrogenase and toxin A antigens when tested by the Triage® *C. difficile* Panel (Microgen New York, NY). In stool samples positive for the glutamate dehydrogenase antigen but negative for the toxin A antigen, the presence of *C. difficile* toxin was confirmed by the cytotoxin assay. Stools were collected from patients diagnosed with CDAD. *C. difficile* was isolated from the stools using the alcohol shock method (Clabots, Gerding et al. 1989). *C. difficile* strains isolated were identified by Gram stain, latex agglutination (MicroAge Byproducts, UK) and colonies that producted apple green fluorescence under long wave UV (365 nm). Toxigenicity of the strains was confirmed by the cytotoxin assay (Alfa, Swan et al. 2002).

2. Bacterial culture methods

Maintenance of *C. difficile* cultures

Frozen stocks of *C. difficile* were maintained in skim milk with 25% glycerol at -70°C. Bench cultures were obtained by sub-culturing frozen stocks to Blood Agar (BA) (Oxoid Nepean, ON) a minimum of two times and inoculating 10 mL of pre-reduced Fastidious Anaerobic Broth (FAB) ((International Diagnostics Group, Bury, England). Cultures in FABs were grown at 37°C in an ambient air incubator for 24 hours and kept at room temperature. Bench cultures were kept for 1 month, at the end of the month new FABs were inoculated. FABs were sub-cultured to BA (once) and following 48 hours incubation at 37°C in a Bactron Anaerobic/Environmental chamber (Sheldon Manufacturing Cornelius, OR) sub-cultured to Brain Heart Infusion (BHI) Broth (Difco Oakville, ON) for overnight incubation before analysis.

Analysis of growth kinetics and toxin A/B production in broth culture was performed. Briefly, overnight *C. difficile* cultures in BHI were diluted with BHI to give a turbidity equivalent to a #3 McFarland standard. This gives a *C. difficile* suspension of ~8$\log_{10}$ cfu/mL. The *C. difficile* suspension was diluted to 10^{-3} and 100 µL of this dilution was added to 10 mL of BHI broth (final dilution 10^{5}) for a final count of ~3$\log_{10}$ cfu/mL. All manipulations were performed in a Bactron Anaerobic/Environmental chamber. The BHI broth tube was sealed with a hungate cap (cap with a rubber septum) and removed from the anaerobic chamber. Cultures were incubated in an ambient air incubator at 37°C for up to 80 hours. Aliquots were aspirated through the rubber septum periodically during incubation for analysis by enumeration of total viable *C. difficile*, enumeration of

C. difficile spores, cytotoxin assay and/or tight-junction assay (described below). All culture conditions were repeated in triplicate and results were reported as mean±standard deviation.

C. difficile viable counts

C. difficile viable counts were performed using the spread plate technique on *C. difficile* agar with moxalactam and norfloxacin (CDMN) (Oxoid Nepean, ON). Tests cultures were serially diluted 1:10 and 100 µL of each dilution was plated on CDMN agar and spread over the surface of the plate. Plates were incubated at 37°C in the anaerobic chamber for 48 hours. Plates with 20 to 200 colony forming units (cfu) per plate were enumerated and the cfu/mL was calculated. All viable counts were performed in triplicate and the results reported as the mean±standard deviation.

Alcohol shock to detect *C. difficile* spores

Spores were detected by the alcohol shock technique. An equal volume of 95% ethanol was added to aliquots of culture in BHI (growth curves) or stool (characterization studies) and incubated at room temperature for 40 minutes. After 40 minutes alcohol shock preps were spun for 10 minutes at 14,000 rpm in a microcentrifuge (Eppendorf 541C Toronto, ON) or at 3,500 rpm in an IEC Centra-8R (International Equipment Company, Chattanooga TN). The supernatant was discarded and the pellet resuspended in BHI to the original culture volume. Viable counts of the alcohol shocked suspensions were performed using CDMN agar and incubated at 37°C in an anaerobic chamber. In the stools *C. difficile* spores were identified by growth on CDMN agar and colony

morphology. On CDMN agar *C. difficile* forms round white/grey colonies ~4 mm in diameter with a rough border and appearance. Suspect colonies were confirmed to be *C. difficile* as described.

Spores were enumerated in BHI culture and in stools by the viable count spread plate technique. Plates with 20-200 cfu/plate were counted and the cfu/mL of the original culture aliquot or stool was calculated. Counts were performed in triplicate and the results were reported as the mean±standard deviation.

3. Cell culture techniques

Human foreskin fibroblasts (HFF, ATCC CRL 1635) are a non-transformed cell line obtained from the pooled foreskins of young children. HFF cells were maintained in myeloma media consisting of RPMI-1640 base (MP Biomedicals Irvine, CA) supplemented with 10% fetal bovine serum (Invitrogen Carlsbad, CA), 200mM L-glutamine (Sigma-Aldrich Oakville, ON), and 100 mM sodium pyruvate (Sigma-Aldrich Oakville, ON). To passage HFF cells the adherent cells were lifted from the flask after incubation with 0.05% trypsin (Gibco Carlsbad, CA) at 37°C in 5% CO_2 for 15 minutes. HFF cells were re-suspended in myeloma media and split either 1:2 or 1:3 to a fresh tissue culture flask. Cells were incubated at 37°C in 5% CO_2 until cells formed a confluent monolayer.

For use in the cytotoxin assay 96 well cell culture plates were seeded with a suspension of HFF cells. Briefly, adherent cells were lifted from flasks as described for passaging

the cells. Cells were than stained with Trypan blue (Sigma-Aldrich Oakville, ON). Stained HFF cells were placed in a Fuchs/Rosenthal chamber and visualized at 100X magnification. The four corner squares were counted and the cells/mL were calculated. Every second column of a 96 well cell culture tray was seeded with 150 µL HFF cells (10^5 cells/mL) suspended in myeloma media supplemented with 100X penicillin/streptomycin (MP Biomedicals, Solon, OH) to give a final concentration of 10,000 IU/mL penicillin and 10,000 µg/mL streptomycin. Plates were incubated at 37°C in 5% CO_2 until HFF cells formed a confluent monolayer on the bottom of the well. Trays were used for the cytotoxin assay for up to three weeks after cells reached confluence.

The adenocarcinoma of the colon (Caco2) cell line (ATCC HTB-37) is a transformed epithelial cell line from a male with colon cancer. Caco2 cells are maintained in tissue culture media consisting of RPMI-1640 base supplemented with 20% fetal bovine serum, 200mM L-glutamine, and 100 mM sodium pyruvate. Caco2 cells were passaged when cells formed a confluent monolayer on the bottom surface of a tissue culture flask (~twice weekly). Caco2 cell monolayers were lifted from the flask using 0.25% trypsin (Gibco Carlsbad, CA) that was incubated for 5 minutes at 37°C in 5% CO_2. Caco2 cells were split at a 1:4-1:6 ratio into fresh tissue culture flasks and incubated at 37°C until cells formed a confluent monolayer.

For use in the tight-junction assay Costar® Transwell® cell culture trays (Corning Inc. Corning, NY) were seeded with a suspension of Caco2 cells. Caco2 cells were counted

by trypan blue staining in a Fuchs Rosenthal chamber as for the HFF cells. Caco2 Cells were diluted in tissue culture media supplemented with penicillin/streptomycin (final concentration 10,000 IU/mL penicillin and 10,000 μg/mL streptomycin) to give a final count of 10^5 cells/mL and 500 μL of Caco2 cells were seeded into the apical insert of the Transwell® culture plates. Tissue culture media with penicillin/streptomycin (1,500 μL) was added to the basal side of the insert. Plates were incubated at 37°C in 5% CO_2 until the Caco2 cells formed a confluent monolayer on the insert. Confluence was assessed by taking transepithelial resistance (TER) measurements across the membrane using a Millipore ERS electrode (Millipore Billerica, MA). Monolayers were considered to be confluent when TER measurements were $\geq$400 Ω/cm^2. TER was measured at three separate spots across the membrane and the mean±standard deviation was recorded.

4. Cytotoxin assay for toxin B

To assess the titre of biologically active toxin B in broth cultures, aliquots of the culture were spun at 14,000 rpm. The supernatant was removed and passed through a 0.2 μm syringe filter. The filtered culture supernatant was diluted 1:10 in myeloma media with penicillin/streptomycin. Toxin B in culture supernatants was neutralized by mixing equal parts of a 1:5 dilution of the filtered culture supernatant in myeloma media with penicillin/streptomycin (non-neutralized) or toxin B specific antibody (neutralized) (Techlab Blacksburg, VA) (final dilution of culture supernatant 1:10) and incubated at room temperature for 30 minutes to assess cultures for toxin B specific activity. Confluent HFF cell monolayers in 96 well cell culture trays were inoculated with 50 μL of non-neutralized and neutralization samples. Trays were incubated at 37°C in 5% CO_2

for 48 hours. Wells were assessed for CPE at 24 and 48 hours post inoculation. Wells were positive for CPE if ≥50% of cells showed cell rounding. Broth cultures were positive for toxin B specific biological activity if the sample well was CPE positive and the neutralization well was CPE negative. Bartels® toxin B (Trinity Biotech Company Carlsbad, CA) or Purified toxin B (Techlab Blacksburg, VA, 0.210mg/mL) and myeloma media with penicillin/streptomycin were used as positive and negative controls, respectively, in the cytotoxin assay.

Stools were prepared for the cytotoxin assay by diluting 1:5 in sterile phosphate buffered saline. Diluted stools were spun at 3,500 rpm for 10 minutes and the supernatant was collected and spun for a further 10 minutes at 14,000 rpm. The supernatant was collected and passed through a 0.2 μm syringe filter. Filtered supernatants were diluted 1:2 in myeloma media with penicillin/streptomycin (final dilution 1:10) or anti-toxin B. Confluent HFF cell monolayers were inoculated and incubated as for the *C. difficile* broth cultures.

Biological activity was assayed in both *C. difficile* broth cultures and stools. Briefly, filtered culture supernatants were diluted in myeloma media with penicillin/streptomycin to obtain a 1:10 dilution of the original than serially diluted by 10 in myeloma media with penicillin/streptomycin. 50 μL of each dilution was inoculated onto a confluent HFF cell monolayer and incubated as described above. Titres, defined as the reciprocal of the highest dilution positive for CPE were determined for the samples. All titres were performed in triplicate and the results recorded as the mean±standard deviation.

5. Tight-junction assay for toxin A

C. difficile broth cultures and stools were spun and filtered as for the cytotoxin assay. The tissue culture media in the insert was removed and replaced with 500 µL of a 1:10 dilution (final dilution factor) of the filtered supernatant. The basal media was replaced with fresh tissue culture media with penicillin/streptomycin. Baseline TER readings were read prior to inoculation of the insert. Transepithelial resistance (TER) measurements were taken every 30 minutes for 6 hours or as a single time point after 30 and 300 minutes. Samples were positive for biologically active toxin A if a resistance drop of ≥50% of the baseline was observed within 5 hours post inoculation. All samples were inoculated to triplicate wells and triplicate reading were taken in each well. Results were recorded as the mean±standard deviation of the % baseline of the nine separate readings. Techlab toxin A (Techlab Blacksburg, VA, 0.460 mg/mL) and tissue culture media with penicillin/streptomycin were included in assays as positive and negative controls, respectively.

6. Molecular techniques

Pulsed-Field Gel Electrophoresis

Pulsed field gel electrophoresis (PFGE) typing of strains by *Sma*I restriction was performed as previously described (Alfa, Kabani et al. 2000) at the National Microbiology Laboratory. PFGE profiles were compared and dendrograms showing genetic relationships were compiled using the Bionumeric 4.0 database. PFGE profiles were classified according to the North American Pulsotype (NAP) classification scheme if matches were found.

PCR amplifications and sequencing reactions

PCR amplifications were performed using AmpliTaq Gold in PCR buffer II (*tcdC, tcdR* and *tcdE*) (Applied biosystems, Foster City CA), 3mM $MgCl_2$ and various primer concentrations unless otherwise indicated. Primers used for PCR amplifications are given in Table 1. An annealing temperature of 58°C was used for all PCR reactions.

PCR amplification of the accessory genes and flanking regions for sequencing was performed using primers *cdd1-A/tcdC-3* (*tcdC*), *TPR-1/tcdB-UP* (*tcdR*) and *Lok6/PrimexA* (*tcdE*). Oligonucleotide synthesis and dideoxy cycle sequencing was carried out by the Genomics Core Facility at the NML.

The B1 (*tcdB*) and A3 (*tcdA*) regions were amplified by PCR using the B1C/B2N and A3C/A4N primer pairs respectively (Rupnik, Braun et al. 1997). PCR amplifications were carried out using *Pfu* high fidelity polymerase to amplify the larger regions.

Table 1. Primers used for PCR amplifications

Sequence	Primer	Gene	product (bp)	Reference
TTTCATACATTTGTGCTGGG	Cdd1-A	*tcdC*	699	NML
AATGCATTTTTGATAAACACATTG	tcdC-3			NML
TTCTAGATTTCATAAAAGATAC	TPR-1	*tcdR*		NML
CTGACATATTATGATATTCTTC	tcdB-UP			NML
GTTGTTTAGATTTAGATGAAAAGA	Lok6	*tcdE*		(Rupnik, Braun et al. 1997)
CTTGGTCTAATGCTATATGCGAG	PrimexA			(Hundsberger, Braun et al. 1997)
AGAAAATTTTATGAGTTTAGTTAATAGAAA	B1C	*tcdB*	3,100	(Rupnik, Braun et al. 1997)
CAGATAATGTAGGAAGTAAGTCTATAG	B2N			
TATTGATAGCACCTGATTTATATACAAG	A3C	*tcdA*	3,100	(Rupnik, Braun et al. 1997)
TTATCAAACATATATTTTAGCCATATATC	A4N			
CTTAATGCAAGTAAATACTGAG	cdtB-pos	*cdtB*	510	(Stubbs, Rupnik et al. 2000)
AACGGATCTCTTGCTTCAGTC	cdtB-rev			

Results

The emergence of the hyper virulent NAP1 strain of *C. difficile* in Canada, USA and Europe has led to investigations into the molecular mechanisms involved in the virulence of *C. difficile* (Loo, Poirier et al. 2005; Pepin, Valiquette et al. 2005). Mutations in the *tcdC* ORF have been implicated in increased virulence of the NAP1 strain (Warny, Pepin et al. 2005). In our study we studied a range of *C. difficile* isolates including NAP1-related strains to further elucidate what role the mutations to the *tcdC* gene play in regulating the hyper toxin production observed in the NAP1 strain. Since the *C. difficile* binary toxin has been identified in the NAP1 strains we studied historical clinical isolates to determine the frequency of binary toxin and assess the role of this toxin in outbreaks. Spores play an important role in nosocomial transmission of *C. difficile*. To evaluate the role of sporulation for the NAP1-related strains we assessed the rate and level of spore formation in the NAP1-related and non NAP1-related strains. To further understand the role of spores and toxin in clinical disease, we correlated *in vitro* broth models of *C. difficile* toxin production and sporulation in the stools of patients with CDAD as little data exists describing these phenomena *in vivo*.

1. Growth kinetics and toxin production of historical isolates

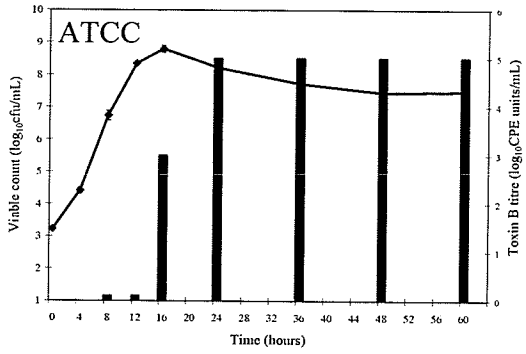
Viable counts and toxin B production

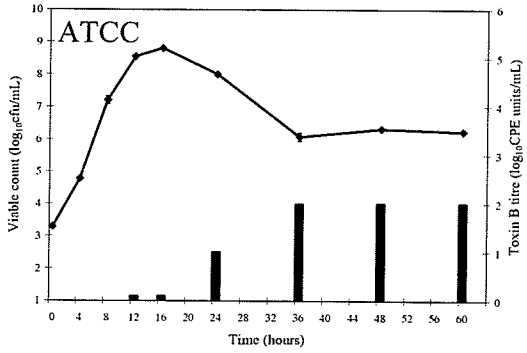
The NAP1 strain of *C. difficile* produces increased toxin levels when compared to clinical isolates (Warny, Pepin et al. 2005). To further examine toxin production in the NAP1 strain the growth kinetics and toxin production were analyzed and compared to clinical isolates of *C. difficile*. The growth dynamics and toxin B production in clinical *C.*

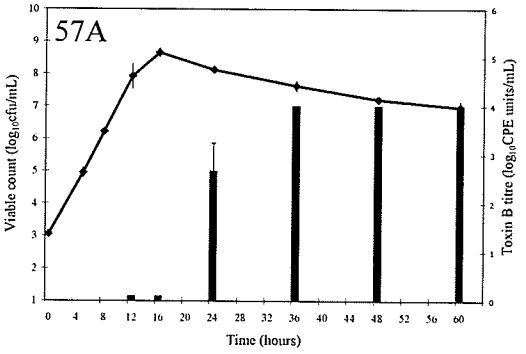
difficile strains 57A (NAP1), 83 (NAP1), 1083, 81A330 and 79A292 were determined and compared to control *C. difficile* strains ATCC 43255 and 43594 which produce high and low levels of biologically active toxins, respectively. Viable bacteria counts and toxin B titres were performed at multiple time points for *C. difficile* cultures grown in BHI broth (Figure 6). Growth curves for all strains were similar. The exponential phase lasted 16 hours with counts peaking at ~9$\log_{10}$ cfu/mL. During the stationary phase (~20-60 hours) viable counts stabilized at ~8$\log_{10}$ cfu/mL. Toxin B titres were assessed during the different phases of the growth curve. Differences in the levels of biologically active toxin B for various strains were observed. Strains were divided into two categories based on toxin titres, hypertoxin (maximum titre >3$\log_{10}$ cpeU/mL) and normal toxin (maximum titre <3$\log_{10}$ cpeU/mL) producers. Three strains 43255 (5$\log_{10}$ cpeU/mL at 24 hours) 57A (4$\log_{10}$ cpeU/mL at 36 hours) and 83 (4$\log_{10}$ cpeU/mL at 36 hours) were designated hypertoxin producers. Strains 81A330, 79A292, 1083 and ATCC 43594 reached maximum toxin B titres of 2$\log_{10}$ cpeU/mL between 36 and 48 hours growth and were classified as normal toxin producers.

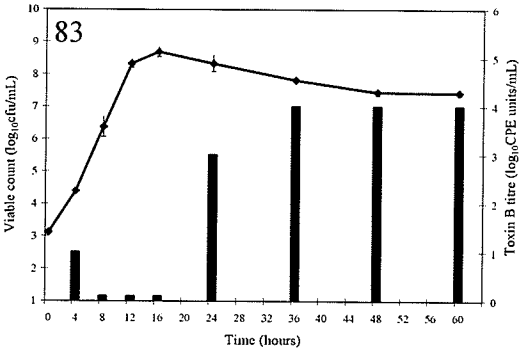
Production of toxin A

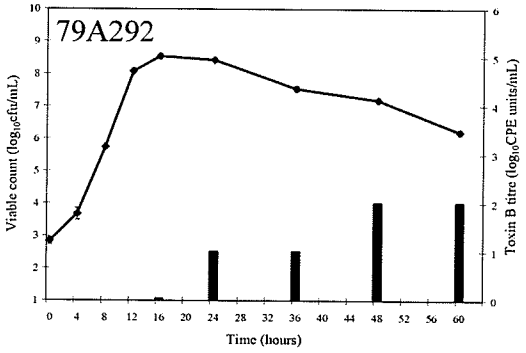
Toxin A biological activity in culture supernatants was quantified using the Caco2 tight-junction assay. Toxin A disrupts the tight-junctions in the Caco2 monolayer causing the depolarization of the monolayer that results in a resistance drop within 300 minutes (Figure 7). Strains were positive for biologically active toxin A if culture supernatants caused a resistance drop of ≥50% (compared to baseline) within 300 minutes. Culture

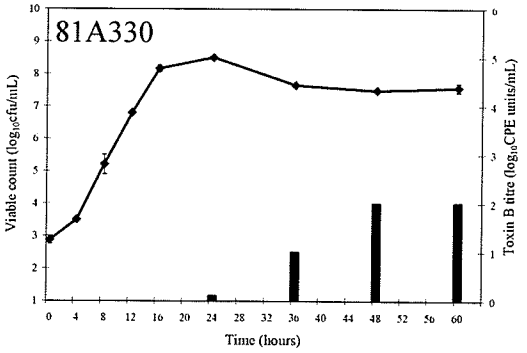

ATCC
Viable count (log_{10}cfu/mL)
Toxin B titre (log_{10}CPE units/mL)
Time (hours)


ATCC
Viable count (log_{10}cfu/mL)
Toxin B titre (log_{10}CPE units/mL)
Time (hours)


57A
Viable count (log_{10}cfu/mL)
Toxin B titre (log_{10}CPE units/mL)
Time (hours)


83
Viable count (log_{10}cfu/mL)
Toxin B titre (log_{10}CPE units/mL)
Time (hours)


79A292
Viable count (log_{10}cfu/mL)
Toxin B titre (log_{10}CPE units/mL)
Time (hours)


81A330
Viable count (log_{10}cfu/mL)
Toxin B titre (log_{10}CPE units/mL)
Time (hours)

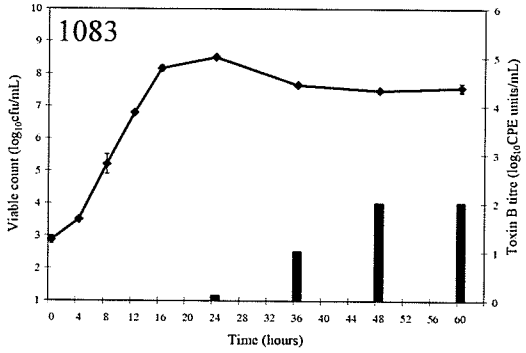

1083
Viable count (log_{10}cfu/mL)
Toxin B titre (log_{10}CPE units/mL)
Time (hours)

Figure 6. Growth kinetics and toxin B production by *C. difficile* isolates used for PaLoc analysis.

The total viable bacteria (vegetative and spores) (line) and toxin B production (black bars) were determined over 300 minutes for isolates grown in BHI broth. Strains evaluated included; ATCC 43255, ATCC 43594, NAP1 clinical strain 57A, NAP1 clinical strain 83, historical clinical strains 79A292, 81A330 and 1083.

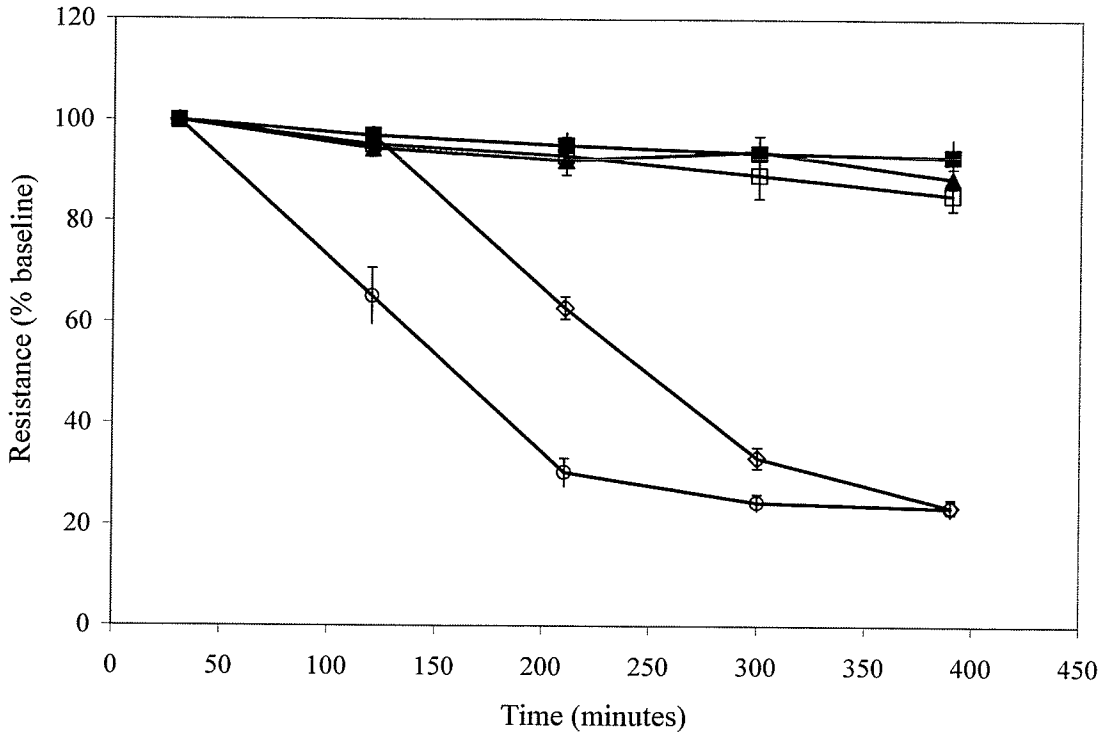


Figure 7. Toxin A induced disruption of the Caco2 monolayer in the tight-junction assay

Titre of biologically active toxin in culture supernatants after 24 hours growth in BHI (see materials and methods). (○) ATCC 43255, (□) 57A, (▲) 81A330, (◊) positive control and (■) negative control were assayed by the tight-junction assay. Results reported as mean±standard deviation of the % baseline resistance in triplicate wells.

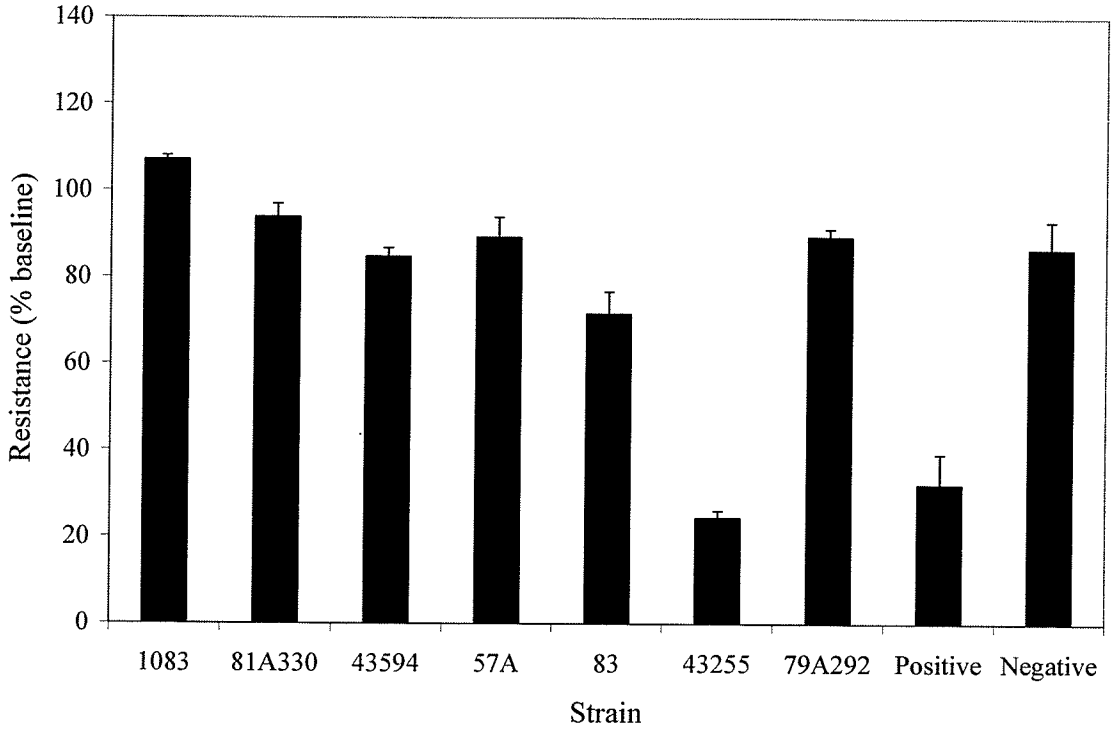
supernatant from the ATCC 43255 strain grown for 24 hours in BHI caused the resistance to drop to 75.6±1.6% of the baseline resistance (Figure 8). After 48 hours growth in BHI broth culture supernatants from the hypertoxin producing strains caused resistance drops to 81.6±0.5 (43255), 71.5±4.2 (57A) and 75.3±1.2% (83) of the baseline resistance (Figure 8). Strain 1083 caused a resistance drop to 52.8±8.1% of the baseline resistance after growing in BHI broth for 48 hours. No biologically active toxin A was detected in strains 81A339, 79A292 and ATCC 43594 (Figure 8).

Conversion of *C. difficile* to the spore form

The efficiency of conversion of vegetative *C. difficile* to spores impacts the ability of *C. difficile* to survive in the environment. The efficiency of conversion to the spore form was quantitated in broth culture using nutrient deprivation to trigger conversion to the spore form. Broth cultures inoculated with standardized low levels of vegetative (~3$\log_{10}$ cfu/mL). *C. difficile* were evaluated and conversion of vegetative *C. difficile* to spores was quantitated by viable counts post alcohol shock. High levels of spores were observed by 48 hours (3-6$\log_{10}$ spores/mL) with all strains showing similar spore levels at 72 hours (~5.5$\log_{10}$ spores/ mL) (Figure 9). Total viable counts were also performed on all cultures to ensure that increased spore levels were not due to increased levels of vegetative *C. difficile* cultures. Total viable counts stabilized at ~7$\log_{10}$ cfu/mL during stationary phase (48 hours) (Figure 9).

The efficiency of conversion to the spore form *C. difficile* strain could have an impact on nosocomial transmission of CDAD. To further characterize the conversion of spores

A


140
120
100
80
60
40
20
0
Resistance (% baseline)
1083
81A330
43594
57A
83
43255
79A292
Positive
Negative
Strain

B

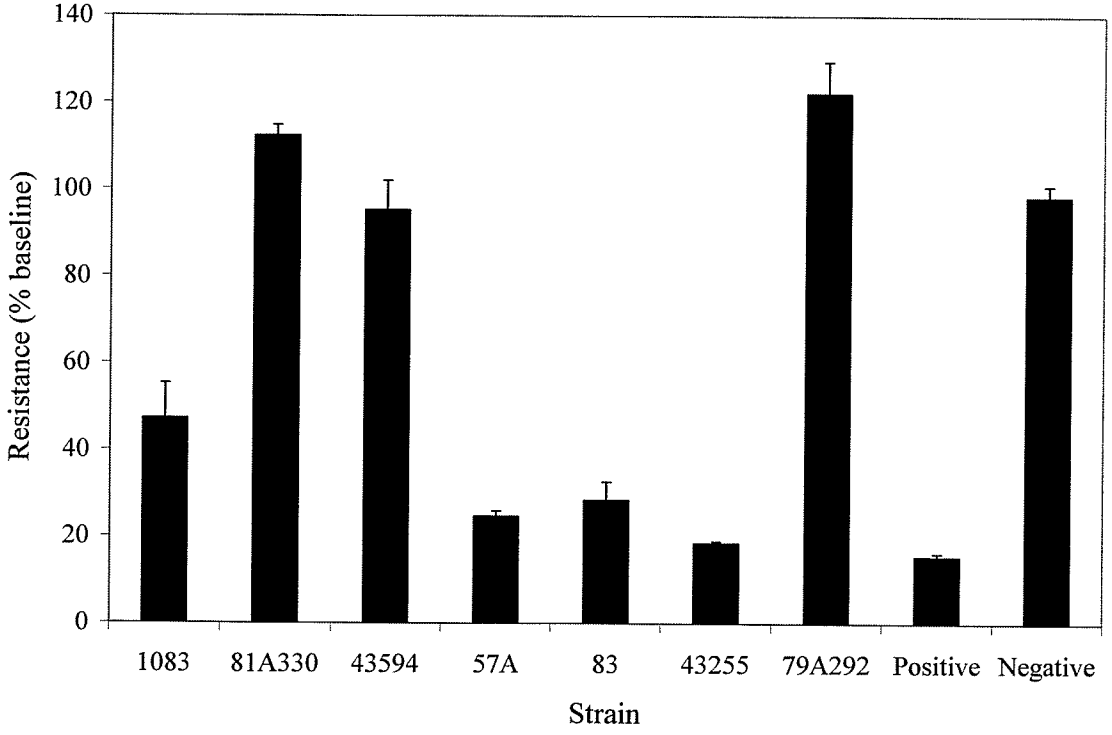
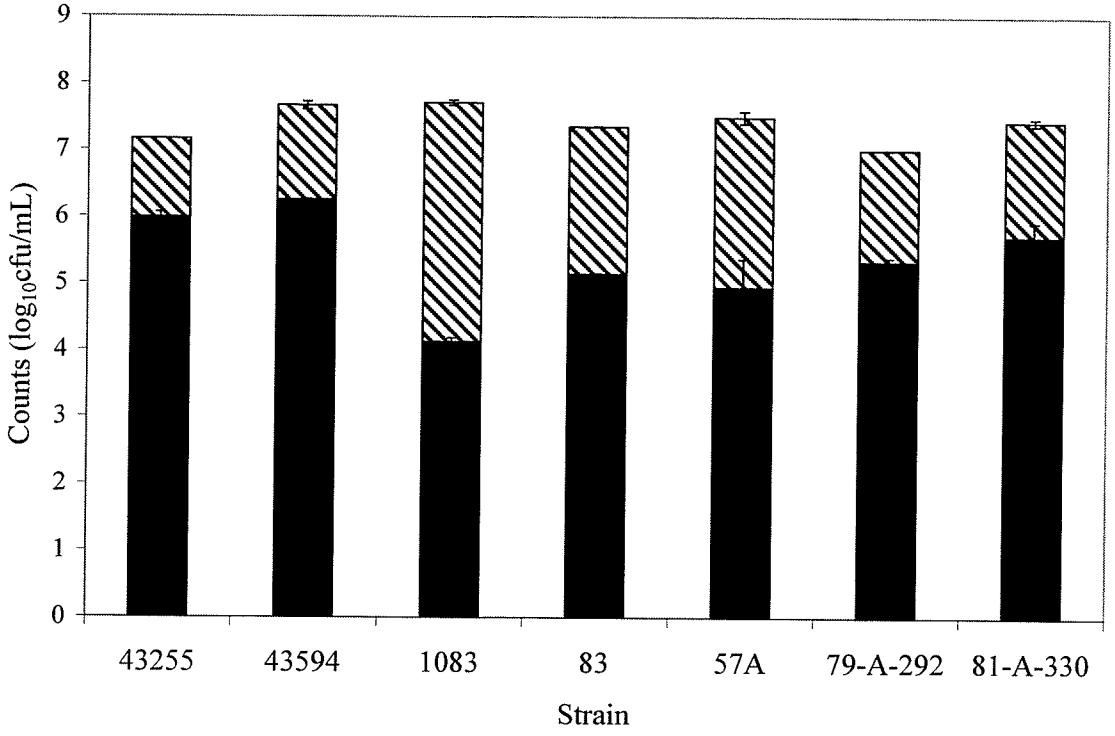

140
120
100
80
60
40
20
0
Resistance (% baseline)
1083
81A330
43594
57A
83
43255
79A292
Positive
Negative
Strain

Figure 8. Toxin A production by *C. difficile* isolates used in PaLoc analysis

Titre of biologically active toxin A after (A) 24 hours and (B) 48 hours incubation in BHI broth. Baseline resistance readings are taken before inserts are inoculated with the culture supernatants (100% baseline). Subsequent measurements were taken at 300 minutes post inoculation and are presented as a percentage of the baseline reading. A culture supernatant was considered positive for biologically active toxin A if a resistance drop greater than 50 % baseline occurred by 300 min. when incubated at 37°C in 5% CO_2.

A


9
8
7
6
5
4
3
2
1
0
Counts (log_{10}cfu/mL)
43255
43594
1083
83
57A
79-A-292
81-A-330
Strain

B

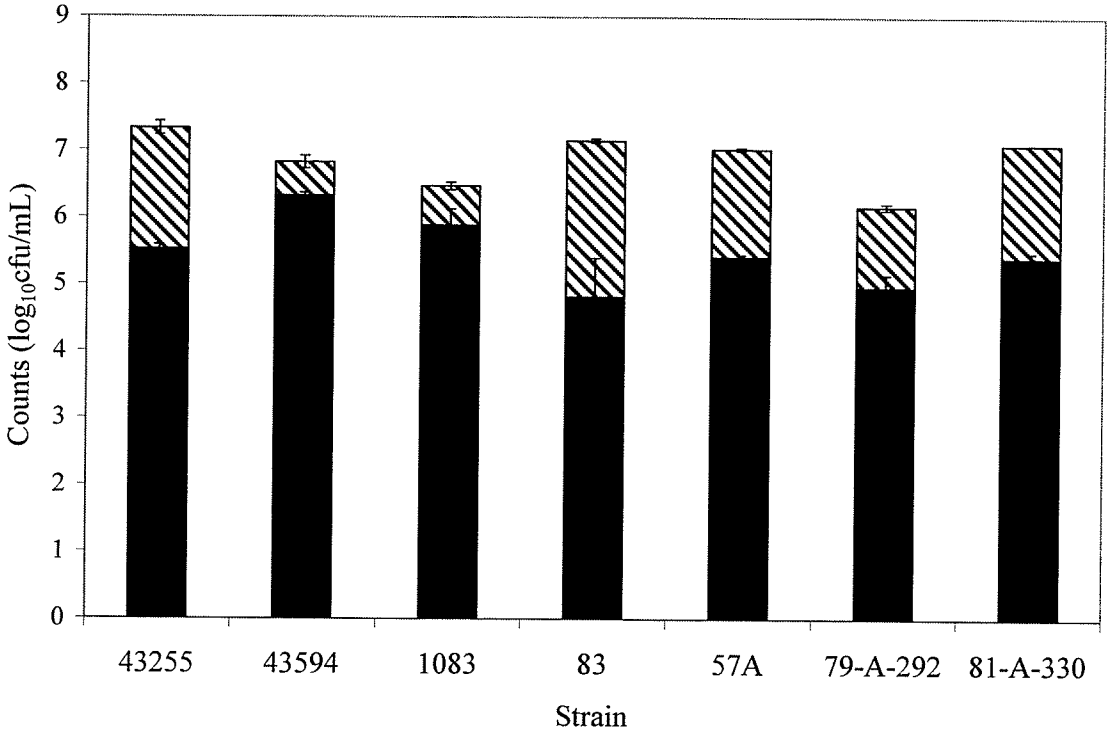

9
8
7
6
5
4
3
2
1
0
Counts (log_{10}cfu/mL)
43255
43594
1083
83
57A
79-A-292
81-A-330
Strain

Figure 9. Enumeration of spores in *C. difficile* isolates used for PaLoc analysis

Spore production by strains of *C. difficile* grown in BHI broth for 48 (A) and 72 hours (B). Solid bars represent spore counts as determined by the alcohol shock method (see Materials and methods). Cross-hatches represent total viable bacteria counts (Vegetative and spores).

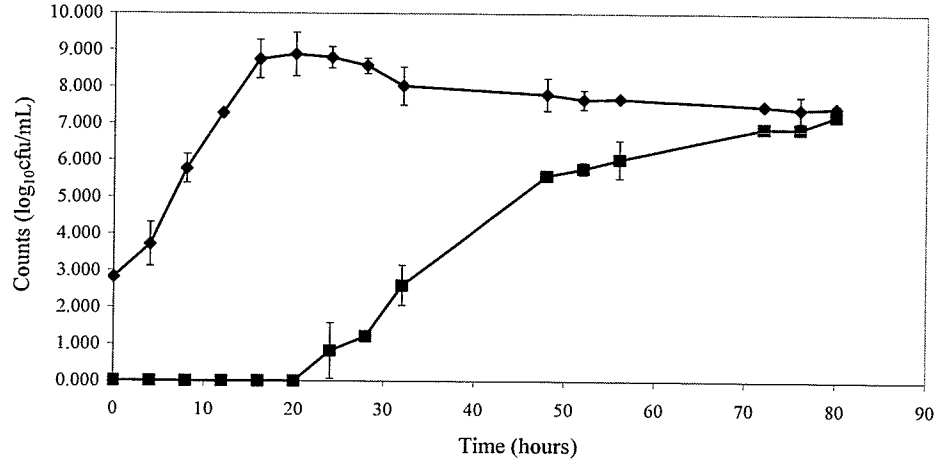
early in broth cultures spore curves were performed on selected isolates, a NAP1 isolate (57A), a clinical isolate (81A330) and a control strain of *C. difficile* (ATCC 43255). Conversion to the spore form was initially detected after 24, 32 and 72 hours for strains 57A, 81A330 and ATCC 43255, respectively, when incubated in BHI broth. Spore levels increased by ~0.25$\log_{10}$ spores/mL/hour (57A), 0.17$\log_{10}$ spores/mL/hour (81A330) and 0.12$\log_{10}$ spores/mL/hour. Maximum spore counts for strains 57A, 81A330 and ATCC 43255 were 7.23±0.06, 4.87±0.09 and 2.15±0.06$\log_{10}$ spores/mL, respectively. Total (vegetative and spore) *C. difficile* counts remained steady at ~7.5$\log_{10}$ cfu/mL during stationary phase (20-80 hours) for all three strains (Figure 10).

2. Molecular characterization of historical isolates

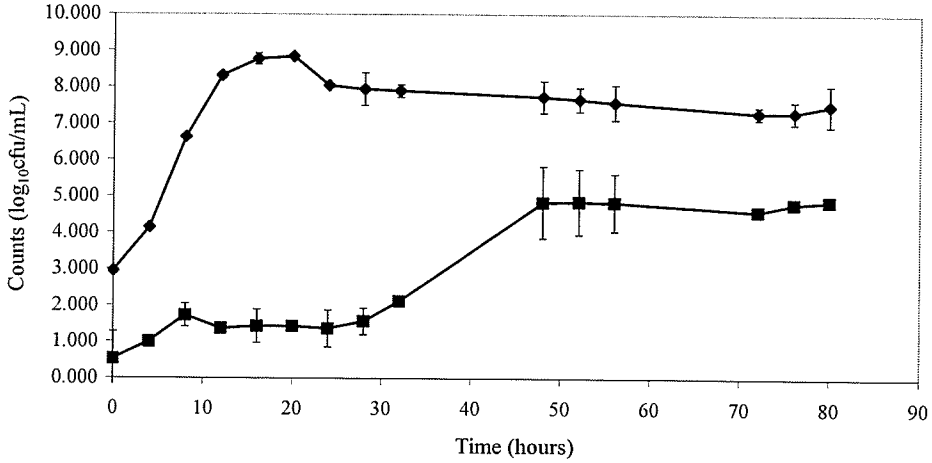
C. difficile toxins

Three toxins have been described in *C. difficile*, toxin A, toxin B and binary toxin. The *tcdA* (A3) and *tcdB* (B1) ORFs were amplified from the NAP1, clinical and control strains to identify any large mutations that could result in altered toxin A or toxin B activity (Rupnik, Braun et al. 1997). PCR amplifications were performed on the two control strains (ATCC 43255 and ATCC 43594) and five clinical isolates (57A, 83, 1083, 81A330 and 79A292). All strains gave a band at ~3.1 kb for fragments A3 and B1 (Table 2) indicating the presence of the toxin A and B genes in the *C. difficile* strains. PCR amplification targeting the binding component of the binary toxin (*cdtB*) was performed to detect binary toxin in the NAP1 strains, clinical isolates and control strains. The presence of the binary toxin in these strains was assessed by amplifying a 510 kb

A ***C.difficile*** **strain 57A**



B ***C.difficile*** **strain 81A330**



C ***C.difficile*** **ATCC strain 43255**

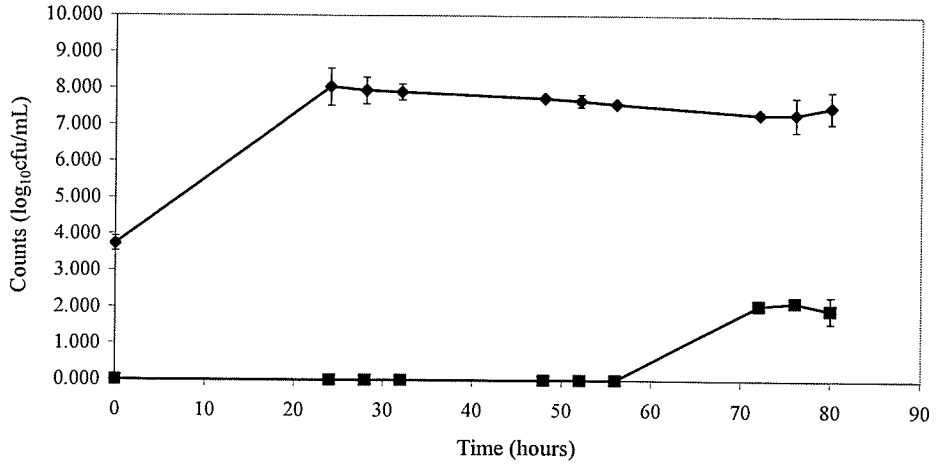


Figure 10. Spore curve for selected *C. difficile* isolates used for PaLoc analysis

Vegetative *C. difficile* (◆) and *C. difficile* spores (■) in broth cultures (BHI) of strains 57A (A), 81A330 (B) and 43255 (C) were measured over 80 hours. Spore conversion was first detected 24 (A), 32 (B) and 72 hours(C) and increased at a rates of 0.35 (A), 0.17 (B) and 0.12 $\log_{10}$spore/mL/hr (C). Each data point represents the mean±standard deviation of three separate experiments.

Table 2. Molecular characterization of *C. difficile* isolates used for PaLoc analysis

Strain	*tcdB* fragment B1 (kb)[1]	*tcdA* fragment A3 (kb)[1]	*tcdR* mutations[2]	*tcdC* allele[3]	*cdtB* PCR[4]
1083	3.1	3.1	None	*tcdC-sc18*	+
ATCC 43255	3.1	3.1	None	Wild type	-
79A292	3.1	3.1	None	*tcdC-sc19*	-
81A330	3.1	3.1	None	*tcdC-sc20*	+
57A (NAP1b)	3.1	3.1	None	*tcdC-sc1*	+
83	3.1	3.1	None	*tcdC-sc1*	+
ATCC 43594	3.1	3.1	None	*tcdC-sc3*	-

[1] PCR reactions previously described (Rupnik, Brazier et al. 2001). Expected products are 2.0 kb (B3) and 3.1 kb (A1).

[2] Predicted mutations based on the comparison of sequence data of isolates to the sequence for the wild-type TcdR (ATCC 43255).

[3] A BLAST search for matching *tcdC* alleles was performed . Alleles up to *tcdC-sc18* have been previously described (Curry, Marsh et al. 2007). New alleles were assigned to *tcdC* genes where no allelic matches were found.

[4] +, positive, presumed to produce binary toxin.

-, gene segments not amplified by PCR, presumed not to produce binary toxin (Stubbs, Rupnik et al. 2000).

region of the *cdtB* gene. The *cdtB* region was amplified from strains 1083, 81A330, 57A and 83. As such these strains were presumed to be positive for binary toxin (Table 2).

Putative negative regulator (*tcdC*)

Mutations in the *tcdC* gene have been identified in the NAP1 strain. As TcdC plays a role in suppressing toxin production, mutations to the *tcdC* would result in hyper toxin production (Table 2). To analyze the role of *tcdC* mutations in increased toxin production the *tcdC* genes of five historical and two ATCC control strains of *C. difficile* were sequenced to identify any possible mutations that could have an effect on TcdC activity (Figure 11). The wild-type *tcdC* translates to a 232 amino acid protein with a putative transmembrane hydrophobic (TMH) domain from amino acids 30-49 and was characterized from ATCC 43255 (Curry, Marsh et al. 2007). Sequence data from the strains analyzed were compared to the wild-type sequence. Four of the *C. difficile* strains (57A, 83, 79A292 and 81A330) have an 18 base pair in frame deletion (Δ329-347) resulting in an in-frame deletion of 6 amino acids when compared to the wild-type *tcdC*. Strains 57A and 83 have a further 1 base pair deletion (Δ117) which leads to a premature stop codon at positions 187-189, upstream of the 18 base pair deletion. This mutation translates to a predicted truncated protein consisting of the 65 C-terminal amino acids of TcdC. The amino acid sequence from Δ117 to the premature stop codon is altered due to the frame shift mutation interrupting the TMH domain. Strain 1083 has a C to A transversion at position 191 introducing a premature stop codon. The resulting protein is 63 amino acids with a wild-type sequence upstream of the early termination site. Several variations of the *tcdC* gene (alleles) have been described. A BLAST search to identify

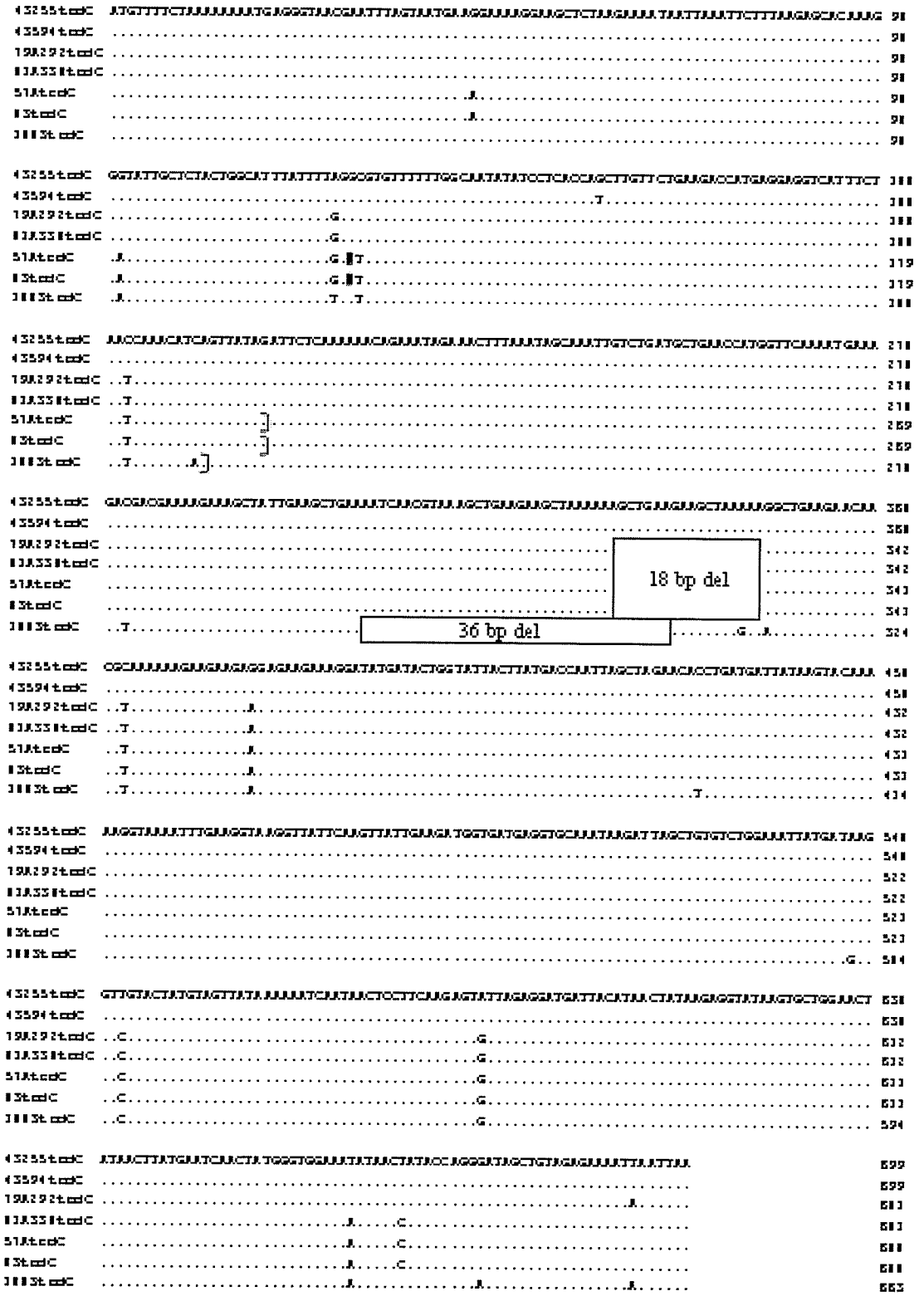

18 bp del
36 bp del

Figure 11. Sequence data for the putative negative regulator (*tcdC*) of *C. difficile* isolates used for PaLoc analysis

The *tcdC* sequences were compared to the wild-type sequence (ATCC 43255). Homologous nucleotides are indicated by dashes while nucleotides differing from the wild-type sequence are indicated. The early stop codons in strains 57A, 83 and 1083 are marked by brackets. Single nucleotide deletions are indicated by filled boxes. Large deletions are indicated by open boxes. Sequence analyses were performed at in the Nosocomial Section of the National Microbiology Laboratory under the supervision of Dr. Michael Mulvey.

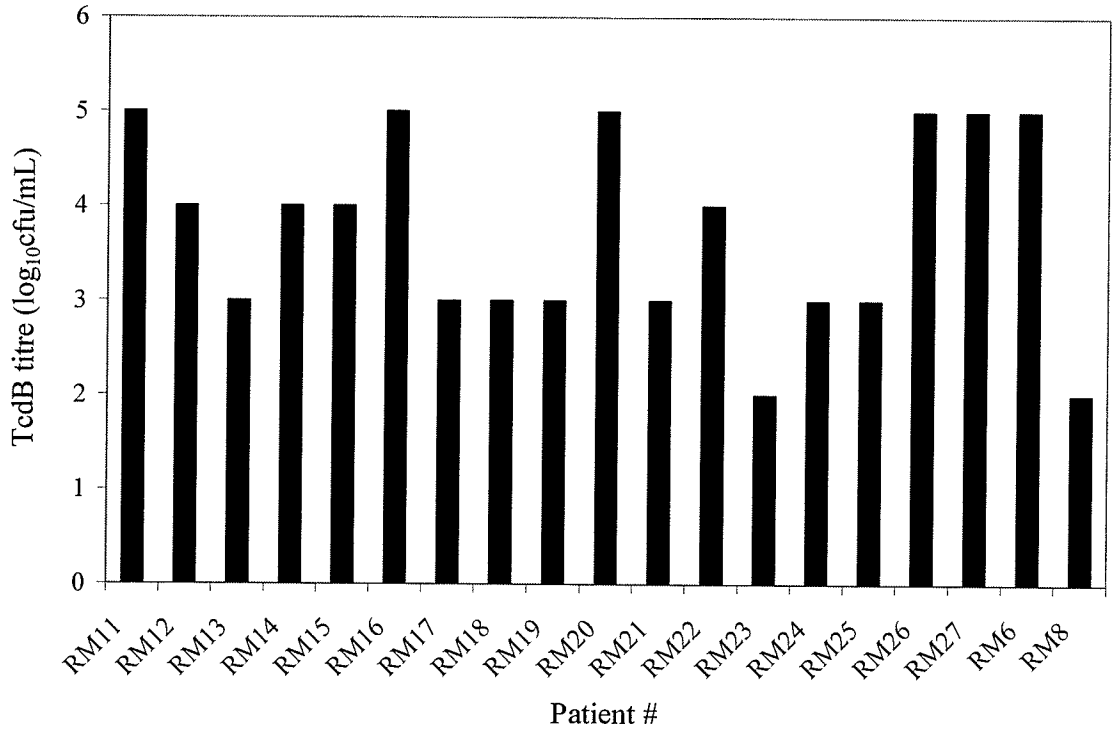
tcdC sequences showing homology to previously described *tcdC* alleles was performed. The sequences of 57A and 83 were identical to the sequence of *tcdC-sc1* previously described (Curry, Marsh et al. 2007) (Table 2). *Sma*I digestion and PFGE on theses strains was performed at the National Microbiology Laboratory. Strains 57A and 83 were typed as NAP1. The remaining strains were unrelated by PFGE (personal communication with Dr. Michael Mulvey).

3. *C. difficile* in stools of patients with CDAD

Toxin B in stool

The growth kinetics and toxin production of various *C. difficile* isolates has been well characterized in broth cultures. However, there have been no studies performed to confirm that the results of these studies can be applied to stool from patients with CDAD. To characterize *C. difficile* toxin production in stool, stools from patients with CDAD were collected and the titre of biologically active toxin B was assessed by the cytotoxin assay within 24 hours of stool collection. The mean toxin B titre in stool was $4\log_{10}$ cpeU/mL (range $2\text{-}5\log_{10}$ cpeU/mL, n=19) (Figure 12, Table 3). To determine whether there was a relationship between toxin in stool and toxin production in broth culture *C. difficile* was isolated from the stool samples as described in the Materials and Methods. Toxingenicity of the *C. difficile* isolates was confirmed by the cytotoxin assay. Broth cultures were inoculated with $\sim3\log_{10}$ cfu/mL of the *C. difficile* and after 48 hours anaerobic incubation the biological activity of toxin B was assessed by the cytotoxin assay. The median toxin B titre in cultures was $3\log_{10}$ cpeU/mL (range $1\text{-}4\log_{10}$

A


TcdB titre (log_{10}cfu/mL)
0
1
2
3
4
5
6
RM11
RM12
RM13
RM14
RM15
RM16
RM17
RM18
RM19
RM20
RM21
RM22
RM23
RM24
RM25
RM26
RM27
RM6
RM8
Patient #

B

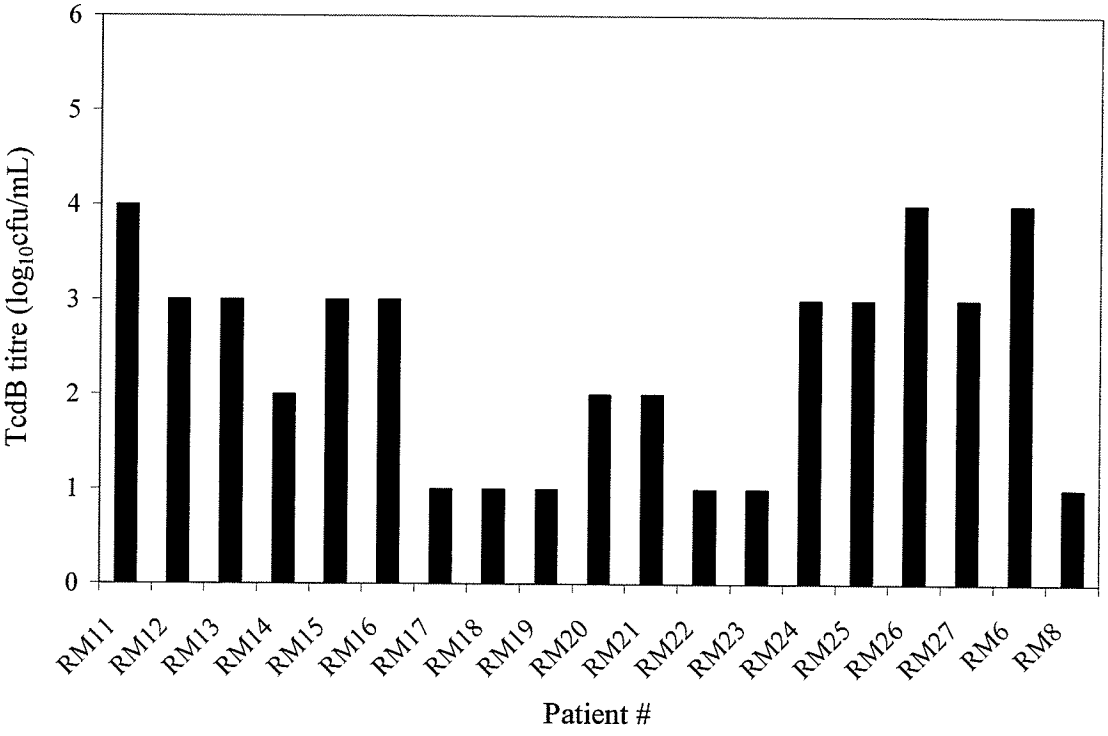

TcdB titre (log_{10}cfu/mL)
0
1
2
3
4
5
6
RM11
RM12
RM13
RM14
RM15
RM16
RM17
RM18
RM19
RM20
RM21
RM22
RM23
RM24
RM25
RM26
RM27
RM6
RM8
Patient #

Figure 12. Titre of biologically active toxin B in stools and *C. difficile* isolates of patients with CDAD

The cytotoxin assay was used to determine the titre of biologically active toxin B in stools (A) and produced in vitro by the clinical isolates (B) (for details see Materials and Methods). Analysis of data using linear regression gave $r=0.7939$, $r^2=0.5683$ and $P=0.0002$ (InStat 3.0 Software).

cpeU/mL, n=19) (Figure 12, Table 3). The sensitivity of the cytotoxin assay was $2X10^{-4}$ ng/mL of purified toxin B.

Toxin A in stool

The *C. difficile* isolates from these stools were grown in broth culture and evaluated for biologically active toxin A. Biologically active toxin A in stool samples of patients with CDAD was undetectable by the Caco2 tight-junction assay (Table 3). The limit of detection for toxin A in the Caco2 tight-junction assay was 7.2 ng/mL (evaluated using purified toxin A).

Spores in stool

The presence of high levels of spores in the stools of patients with CDAD would increase the nosocomial transmission CDAD. Fresh stool samples from patients with CDAD were collected and spores were enumerated in the stools by the alcohol shock method. All strains were confirmed as toxigenic *C. difficile*. The median spore count in the stools was $5.84\log_{10}$ spores/mL (range $4.41\text{-}8.16\log_{10}$ spores/mL). Toxin B titres were performed on stools to determine if there is a relationship between toxin and spore levels in the stool (Table 3).

PFGE typing of *C. difficile* clinical isolates from stools

To demonstrate that the *C. difficile* isolates obtained from patients were not one clonal strain circulating in this facility the strains were typed by SmaI restriction PFGE (Alfa, Kabani et al. 2000). Two clusters of isolates based on PFGE profiles were identified,

NAP2 (13 isolates) and 0122 (6 isolates) (Figure 13, Table 3). Isolates RM14 and RM26 had PFGE patterns matching the NAP1 epidemic strain. The remaining isolates evaluated were unrelated by PFGE (Figure 13).

4. Prevalence of binary toxin and *tcdC* mutations in clinical strains of *C. difficile*

The NAP1 strain has been implicated in epidemics of *C. difficile* worldwide. The binary toxin produced by the NAP1 strain is believed to increase the pathogenicity of the NAP1 outbreak strain. Sequence analysis of the *tcdC* gene and PCR amplification of *cdtB* was performed on 40 clinical isolates of *C. difficile* obtained from 2000-2001. During this time this hospital experienced an outbreak of CDAD with two clonal strains isolated (based on PFGE profiles). The 40 clinical isolates selected for analysis were representative of the PFGE profiles of the *C. difficile* isolates from this period. Eight different *tcdC* alleles were identified (Figure 14). A BLAST search revealed that alleles *tcdC-sc3*, *tcdC-sc9*, *tcdC-sc7*, *tcdC-sc13* and *tcdC-sc1* have been previously described (Curry, Marsh et al. 2007). Two novel *tcdC* alleles, *tcdC-rm1* and *tcdC-rm2*, were identified. Three *C. difficile* strains with a *tcdC* allele matching the NAP1 allele (*tcdC-sc1*) were identified. The *tcdC-sc1* allele is characterized by a 1bp deletion (Δ117) causing a frame-shift in the ORF resulting in an early stop codon. The *tcdC-rm1* allele had an in-frame 18 bp deletion in the oligonucleotide repeat region resulting in the loss of 6 amino acids in the translated product. Other mutations observed were either silent mutations or resulted in conserved amino acid substitutions in the translated protein product. The 510 bp *cdtB* PCR product was amplified from all three strains with the *tcdC-sc1* allele and both of the *tcdC-sc13* strains (Table 4).

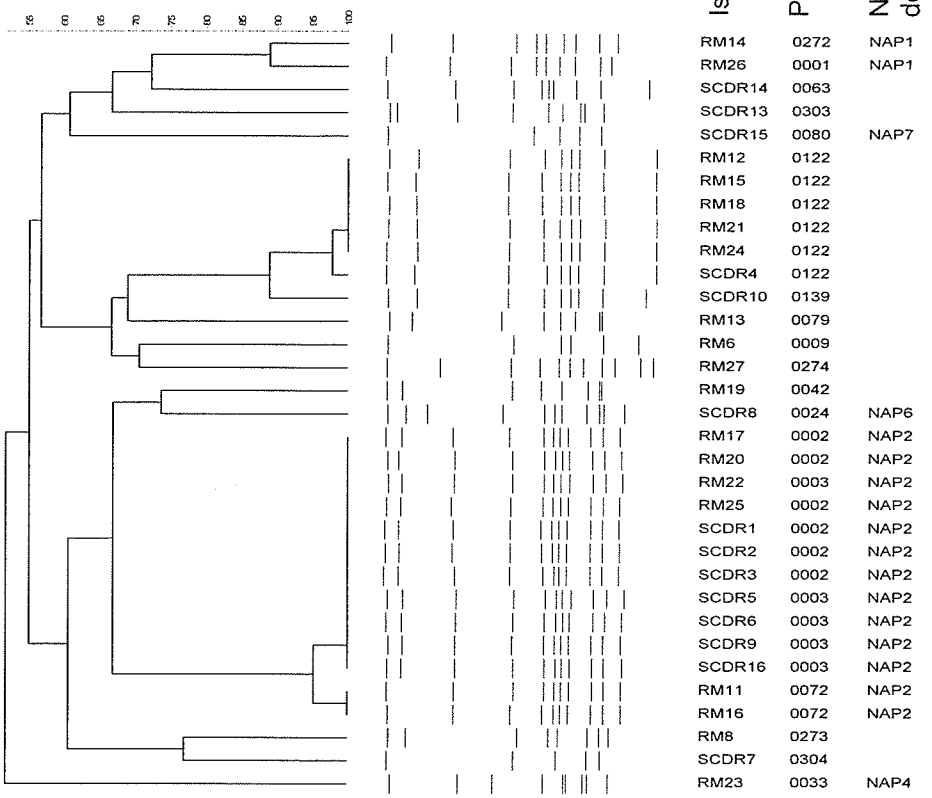
Table 3. Characterization of stools and *C. difficile* isolates from patients with CDAD

Strain	Spores [log_{10}cfu/mL]	toxin B titre in stool [log_{10}cpe/mL]	Toxin A stool[1]	toxin B titre in broth culture [log_{10}cpe/mL]	Toxin A broth culture[1]	*Sma*I PFGE type[2]
RM6	n.d.	5	-	4	+	0009
RM8	n.d.	2	-	1	-	0278
RM11	n.d.	5	-	4	-	0072
RM12	n.d.	4	+	3	-	0122
RM13	n.d.	3	-	3	-	0079
RM14	n.d.	4	-	2	-	0272
RM15	n.d.	4	-	3	-	0122
RM16	n.d.	5	-	3	-	0072
RM17	n.d.	3	-	1	+	0002
RM18	n.d.	3	+	1	-	0122
RM19	n.d.	3	-	1	-	0042
RM20	n.d.	5	-	2	-	0002
RM21	n.d.	3	-	2	-	0122
RM22	n.d.	4	-	1	-	0002
RM23	n.d.	2	-	1	-	0033
RM24	n.d.	3	-	3	-	0122
RM25	n.d.	3	-	3	-	0002
RM26	n.d.	5	-	4	-	0001
RM27	n.d.	5	-	3	-	0274
SCDR5	6.87	4	n.d.	3	n.d.	0003
SCDR6	5.76	5	n.d.	2	n.d.	0003
SCDR7	4.41	3	n.d.	2	n.d.	0304
SCDR8	5.13	3	n.d.	1	n.d.	0024
SCDR9	8.16	5	n.d.	2	n.d.	0003
SCDR10	6.45	5	n.d.	2	n.d.	0139
SCDR13	6.07	4	n.d.	2	n.d.	0303
SCDR14	5.27	4	n.d.	2	n.d.	0063
SCDR15	5.91	4	n.d.	4	n.d.	0080
SCDR16	5.69	1	n.d.	2	n.d.	0003

[1] A 1:5 dilution of the stools or of culture supernatant from broth cultures of *C. difficile* isolates was mixed 1:2 with tissue culture media and inoculated onto a transwell insert as described in the Materials and methods. + indicates ≥7.2 μg/mL toxin A.

[2] *Sma*I PFGE analysis performed at the National Microbiology Laboratory under the supervision of Dr. Michael Mulvey. PFGE types 0001 and 0272 are both NAP1 types. Types 0002 and 0072 are NAP2. Type 0033 is NAP4.

n.d. Parameter not tested for this sample.



Isolate #	Profile #	NAP designation
RM14	0272	NAP1
RM26	0001	NAP1
SCDR14	0063	
SCDR13	0303	
SCDR15	0080	NAP7
RM12	0122	
RM15	0122	
RM18	0122	
RM21	0122	
RM24	0122	
SCDR4	0122	
SCDR10	0139	
RM13	0079	
RM6	0009	
RM27	0274	
RM19	0042	
SCDR8	0024	NAP6
RM17	0002	NAP2
RM20	0002	NAP2
RM22	0003	NAP2
RM25	0002	NAP2
SCDR1	0002	NAP2
SCDR2	0002	NAP2
SCDR3	0002	NAP2
SCDR5	0003	NAP2
SCDR6	0003	NAP2
SCDR9	0003	NAP2
SCDR16	0003	NAP2
RM11	0072	NAP2
RM16	0072	NAP2
RM8	0273	
SCDR7	0304	
RM23	0033	NAP4

Figure 13. PFGE profiles of *C. difficile* isolates from stools of patients with CDAD

Isolates from stool samples were analyzed by *Sma*I restriction and separated by PFGE. PFGE was performed as previously described (Alfa et al. 2000) in the Nosocomial section of the National Microbiology Laboratory under the supervision of Dr. Michael Mulvey.

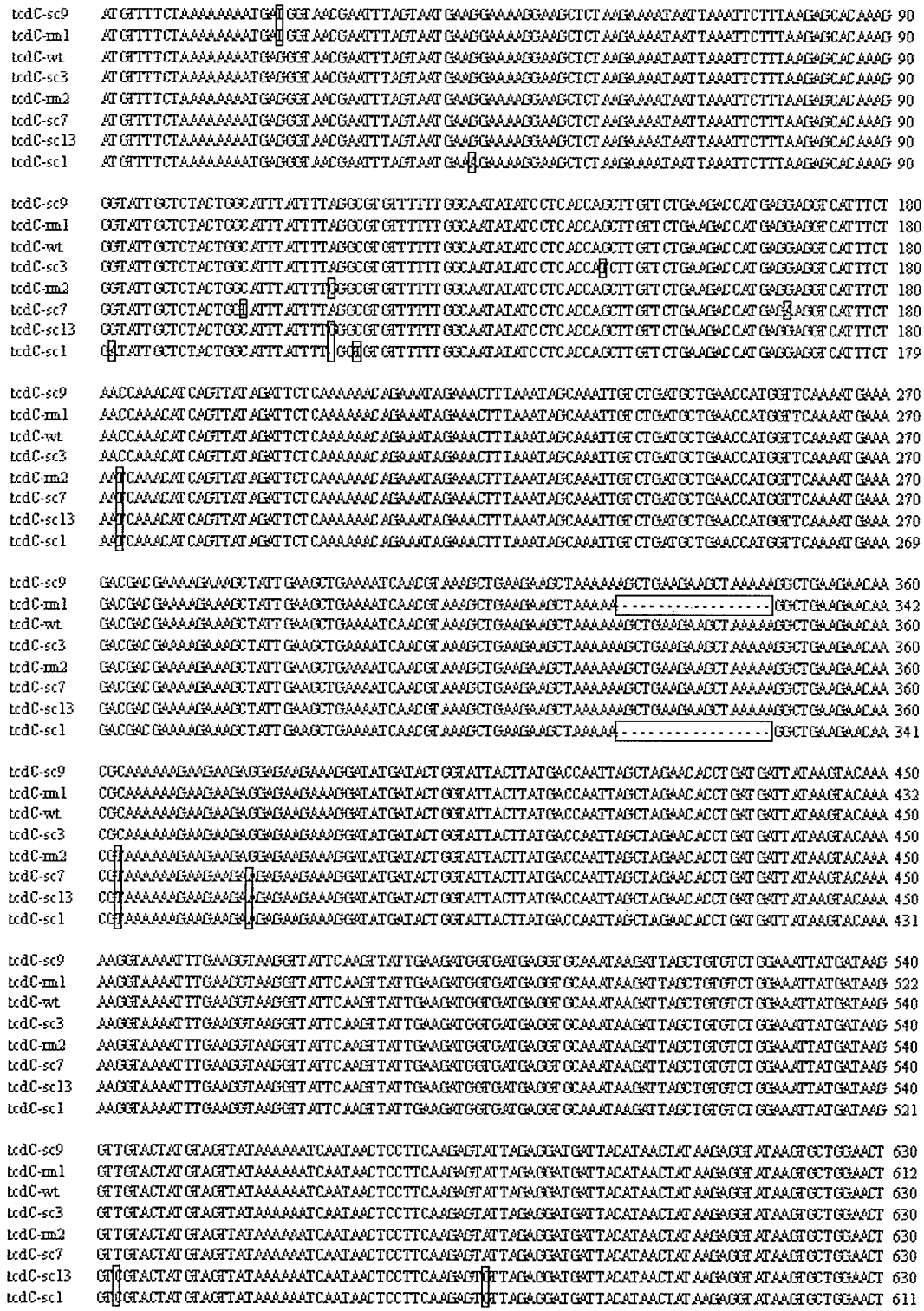

tcdC-sc9
tcdC-rm1
tcdC-wt
tcdC-sc3
tcdC-rm2
tcdC-sc7
tcdC-sc13
tcdC-sc1

Figure 14. Sequences of representative *tcdC* alleles from *C. difficile* isolates used for the determination of the frequency of binary toxin

Representative *tcdC* sequences for 40 clinical isolates of *C. difficile*. A BLAST search was performed to identify *tcdC* alleles. Alleles *tcdC-sc1* - *tcdC-sc18* have been previously described (Curry 2007), alleles *tcdC-rm1* and *tcdC-rm2* were unique to this study. Sequences were compared to the wild-type sequence (ATCC 43255). Any sequence variations are shown by an open box.

Table 4 Frequency of *tcdC* mutations in clinical strains

tcdC allele[1]	Truncating Mutation	Protein length (aa)	Allele frequency (n=40)	*cdtB*[2]
tcdC-sc3	None	None	14	0
tcdC-sc9	None	None	10	0
tcdC-rm1	Δ329-347	226	1	0
tcdC-wt	None	None	3	0
tcdC-rm2	None	None	3	0
tcdC-sc7	None	None	4	0
tcdC-sc13	None	None	2	2
tcdC-sc1	Δ117	65	3	3

[1] tcdC-sc1to tcdCsc18 alleles have been previously described (Curry, Marsh et al. 2007). *tcdC-rm1* and *tcdC-rm2* were unique to this study.

[2] *cdtB* amplified as previously described (Stubbs, Rupnik et al. 2000). Strains that *cdtB* was amplified from were presumed to be positive for binary toxin.

Discussion

Our data demonstrated that the NAP1 strain of *C. difficile* produces increased levels of biologically active toxins A and B when compared to non-NAP1 strains. The data from our study is in accordance with a previous study (Warny, Pepin et al. 2005) that found that the NAP1 outbreak strain produces 16 and 23X (in μg/L) more toxin B and toxin A, respectively, than other clinical strains (toxinotype 0). The growth kinetics and toxin production for five unique (by PFGE) clinical isolates were compared to *C. difficile* strains that produce high (ATCC 43255) and normal (ATCC 43594) levels of biologically active toxin. Strains were subdivided into two categories based on the levels of biologically active toxin B, as determined by the cytotoxin assay, in the supernatants of broth cultures. The two NAP1-related and the ATCC 43255 strains were hyper toxin producers. The hyper toxin producing strains reached maximum toxin B titres of $4\log_{10}$ cpeU/mL (57A, 83) and $5\log_{10}$ cpeU/mL (ATCC 43255) by 24-36 hours. Conversely, strains 1083, 79A292, 81A330 and ATCC 43594 were normal toxin producers. Normal toxin producers reached maximum toxin B titres of $2\log_{10}$ cpeU/mL at 36-48 hours. In further support of increased toxin production by the NAP1-related strains, biologically active toxin A was detectable after 48 hours for hyper toxin producing strains but not for normal toxin producing strains of *C. difficile* when assessed by the Caco2 tight-junction assay. The only exception to this was strain 1083. The supernatant from 48 hour broth culture of strain 1083 was able to induce a resistance drop of 52.8±8.11% from baseline resistance indicating a low level of toxin A in the culture. No toxin A was detected in the culture supernatants from the remaining normal toxin producing strains. The recent

emergence of the NAP1 epidemic *C. difficile* strain has led to increased investigation into the molecular mechanisms regulating the production of the *C. difficile* toxins.

Our data supports previously reported data (Warny, Pepin et al. 2005) that the NAP1-like strains produce increased levels of toxin, despite differences in experimental design of the previous study. We measured levels of biologically active toxin in broth culture using the cytotoxin assay while previous data measured concentrations in μg/L (Warny, Pepin et al. 2005). However, our data refutes current reports that mutations to the TcdC are fully responsible for the increased toxin production by the NAP1 strain (Warny, Pepin et al. 2005; MacCannell, Louie et al. 2006; Curry, Marsh et al. 2007; Matamouros, England et al. 2007). In our study the two NAP1-like strains (57A and 83) produced $2\log_{10}$ cpeU/mL greater toxin B and in broth culture as compared to non-NAP1-like strains (79A292 and 81A330). Strain 1083, that has a truncating mutation in the *tcdC* gene, produced low levels of biologically active toxin B, similar to the levels of toxin B produced by the normal toxin producing control strain (ATCC 43594). However, the ATCC 43255 strain, which has an intact *tcdC* gene (Hundsberger, Braun et al. 1997), produced increased levels of biologically active toxin when compared to the NAP1-like strains. The TcdC protein has been putatively identified as a negative transcriptional regulator of the PaLoc ORFs (Hundsberger, Braun et al. 1997). An 18 bp deletion to the *tcdC* ORF was described in the NAP1 epidemic strain in a study examining the growth and toxin production. Warny et al (2005) reported that the NAP1 strain produced 16X and 23X more toxin B and toxin A, respectively, compared to toxinotype 0 clinical isolates. The increased toxin production was attributed to the mutation in the *tcdC* ORF.

We also found that the NAP1 strain produced higher titres of toxins A and B in broth culture. Measuring levels of biologically active toxin rather than μg/L amounts allows for the analysis of results as they pertain to the effects of increased biological activity of the toxin, not simply increased amount of toxin. An increase in the μg/L toxin of a biologically inactive toxin would not necessarily translate to an increase in virulence.

The data from our experiments do not support the assertion that mutations to the *tcdC* are the sole factor in the hyper toxin production in the NAP1 strain of *C. difficile*. Putatively, truncation of TcdC causes increased toxin production in the NAP1 strain of *C. difficile*. Current evidence indicates that ATCC 43255 is a hyper toxin producing *C. difficile* strain (Soehn, Wagenknecht-Wiesner et al. 1998; Karlsson, Burman et al. 1999; Akerlund, Svenungsson et al. 2006). Our data demonstrate that the ATCC strain produces levels of biologically active toxin B that exceed those produced by the NAP1 hyperproducer strains and biologically active toxin A is detectable in culture supernatants from 24 hour broth cultures of *C. difficile* ATCC 43255. This is an anomaly when compared to other toxinotype 0 clinical isolates (in μg/L) (Warny, Pepin et al. 2005) or to 19 clinical isolates (in cpeU/mL) grown in broth culture. The two ATCC control strains (43255 and 43594) produce homologous TcdC proteins. However, *C. difficile* ATCC 43594 produces $2\log_{10}$ cpeU/mL less toxin B than the ATCC 43255 strain. Toxin production by *C. difficile* strain 1083, which produces a 63 amino acid TcdC, was $2\log_{10}$ cpeU/mL lower then in the NAP1 strains despite the truncated TcdC. This finding would seem to indicate that truncation of the TcdC alone is not sufficient to induce hyper toxin production. This provides evidence that TcdC has a limited impact on the overall levels of toxin

production in broth cultures. Our data, that a toxinotype 0 *C. difficile* (wild-type *tcdC* gene) produces high levels of toxin while a strain of *C. difficile* with a truncated *tcdC* produces normal levels of toxin in broth culture, implies that other factors influence toxin production.

No variations were found in the remaining PaLoc ORFs that could fully explain the hyper toxin production by the NAP1 strain. Research to date has focused on the characterization of *tcdC* and the role of mutations in this gene in toxin production. However, to the authors' best knowledge, no information is available on the molecular characteristics of the remaining PaLoc ORFs and how these genes relate to toxin production in the NAP1 strain. The PaLoc from the *C. difficile* type strain, ATCC 43255, was amplified and the PaLoc ORFs were sequenced and has been used as a model for the wild-type PaLoc (Hundsberger, Braun et al. 1997). The PaLoc accessory proteins are essential in the current model for the molecular regulation of toxin transcription. No mutations in the *tcdR* ORF were identified that could fully explain the quantitative differences in toxin A and toxin B produced by NAP1 versus non-NAP1-like strains, when sequence data was analyzed. The *tcdE* codes for a holin-like protein that is putatively involved in toxin secretion by *C. difficile* (Hundsberger, Braun et al. 1997). Strain 1083 had a frame-shift mutation that introduced an early stop codon in *tcdE*. The translated product to an 18 amino acid C-terminal truncated TcdE. The truncated TcdE is most likely inactive which would interfere with toxin secretion by *C. difficile*. However, in the *tcdE* ORF there is a second start codon a few nucleotides down from the early stop codon. If TcdE can be translated from the second start codon the N-truncated protein

produced that retain functionality on its own or by associating with the C-terminal truncated protein. Amplicons corresponding to the 3.1 kb targets of the A3 and B1 regions of the *tcdA* and *tcdB* ORFs were obtained from all the clinical isolates and control *C. difficile* strains used to study the growth kinetics and toxin production. While we were unable to detect any genetic differences in the PaLoc accessory genes that could account for the differences in *in vitro* toxin production observed in our strains there could be other factors that influence toxin production.

The study of pathogenesis in the NAP1 strain of *C. difficile* has been focused primarily on the effects of mutations in the PaLoc (specifically to the *tcdC* ORF). While this likely contributes to the hyper toxin production observed in the NAP1 there are many virulence factors that influence the pathogenesis of *C. difficile*.

While our study showed that *tcdC* truncation cannot be the only factor influencing toxin hyper production it was unable to identify other factors involved in toxin regulation that could affect toxin production. The growth kinetics and toxin production of *C. difficile* isolates from diverse genetic backgrounds with varying mutations to the *tcdC* ORF, but an otherwise intact PaLoc, demonstrated that while truncating mutations to the *tcdC* ORF could cause hyper toxin production in the NAP1 strain, they could not fully explain increased toxin production in the NAP1 strains. Further, toxin B production was detected late in logarithmic growth in the NAP1 isolates and in early stationary phase for the other clinical isolates. This supports previous studies (Freeman, Baines et al. 2007) that reported toxin production occurred earlier in NAP1 strains. However, the ATCC control

strain (43255), which possesses the wild-type *tcdC* gene (Curry, Marsh et al. 2007), produced higher levels of toxin A and B in broth culture than the NAP1 strains analyzed. Also, toxin was detected earlier in supernatants from broth cultures of *C. difficile* ATCC 43255 than in the NAP1 strains. The *C. difficile* isolate, 1083, also had a truncating mutation in the *tcdC* ORF, however this isolate did not produce high titres of biologically active toxin in broth culture. In the current model for the transcriptional regulation of toxin production the three PaLoc accessory genes control the initiation (*tcdR*) and suppression (*tcdC*) of transcription and the secretion of toxin (*tcdE*). The *tcdC* ORF is transcribed during early logarithmic growth when the other PaLoc genes are repressed and thus the protein product was identified as a putative negative transcriptional regulator (Hundsberger, Braun et al. 1997). More recent evidence demonstrates that TcdC binds to the alternative sigma factor coded on the *tcdR* ORF indirectly inhibiting transcription from the toxin promoters. Thus, there must be other factors influencing virulence in the NAP1 epidemic strain.

In this study no alterations in the toxin A and B genes were identified that could explain the higher levels of biologically active toxin produced by the NAP1 strain. PCR amplification of the B1 (*tcdB*) and A3 (*tcdA*) regions was unable to detect any large deletions to the toxin A and toxin B genes. Polymorphisms in the toxin genes have been identified in isolates of *C. difficile* (Rupnik, Braun et al. 1997). A toxinotyping scheme to identify and characterize these polymorphisms, as well as other variations in the PaLoc, has been established (Rupnik, Avesani et al. 1998). The contribution of variant toxinotypes to the virulence of *C. difficile* isolates has been disputed. While some

toxinotypes do not produce detectable toxin, usually as a result of an incomplete PaLoc, others such as toxinotype III (NAP1) have been shown to produce high levels of biologically active toxin. Toxinotype 0 was described from ATCC 43255 as the *C. difficile* type strain. However, amongst toxinotype 0 isolates ATCC 43255 is an anomaly, producing significantly higher levels of toxin than most other clinical toxinotype 0 isolates (Warny, Pepin et al. 2005).

The data presented in this study demonstrated that biologically active toxin B produced in broth culture correlates to toxin levels in the stools of patients with CDAD. While research to date (Warny, Denie et al. 1995) has established the hyper production of toxins by the NAP1 strain in broth culture it is unclear whether this translates to the presence of increased toxin levels in patients with CDAD due to NAP1. We identified a linear relationship between toxin B production in stools and of isolates grown in broth culture with an approximate 10-fold increase in toxin titres in stools. Following this trend the NAP1 strain would produce ~5$\log_{10}$ cpeU/mL in stool. A previous study (Akerlund, Svenungsson et al. 2006) was unable to demonstrate a correlation between toxin B levels in stools of patients with CDAD and of isolates in broth culture when toxin levels were assessed using a McCoy cell cytotoxin assay. The focus of research on the NAP1 strain of *C. difficile* has been the molecular characterization of the *tcdC* ORFs and how it affects toxin production in broth culture. Severity of CDAD, as characterized by increased frequency of diarrhea, has been shown to correlate to increased toxin in stool (Akerlund, Svenungsson et al. 2006). However, this study was unable to correlate

increased toxin in stool to the more severe manifestation of CDAD, PMC. As such, the importance of increased toxin production in disease severity is not clearly understood.

We were unable to detect biologically active toxin A in stools or in broth culture of isolates by the Caco2 tight-junction assay. This was unexpected as Toxin A and B are transcribed as monocistronic or polycistronic transcripts from P*tcdB* or P*tcdA*. This would result in a greater number of *tcdA* transcripts than *tcdB* transcripts. As such toxin A would be expected to be expressed in higher quantities than toxin B. Previous studies have reported concentrations of 4 μg/L (i.e. 4 ng/mL) toxin A in broth culture (Warny, Pepin et al. 2005). We determined the limit of detection of the Caco2 assay to be 7.2 ng/mL of purified toxin A. As such it is possible that the levels of biologically active toxin A in stools and in broth culture are below the level of detection of the Caco2 assay system. The cytotoxin assay is much more sensitive as the limit of detection for purified toxin B was 2×10^{-4} ng/mL, facilitating sensitive detection of toxin B levels present in broth culture.

The NAP1 strain of *C. difficile* produced increased biologically active toxin A and B earlier in the growth phase (*in vitro*) when compared to non-NAP1 strains. Two isolates from the stools of patients with CDAD were identified as NAP1-subtypes by *Sma*I restriction PFGE (Alfa, Kabani et al. 2000). These isolates had $4\log_{10}$ and $5\log_{10}$ cpeU/mL toxin in the stools. The mean toxin B titre in the stools of patients with CDAD was $4\log_{10}$ cpeU/mL (range 2-$5\log_{10}$ cpeU/mL). This is evidence that the NAP1 isolates produce toxin B levels in the upper range of toxin B titres observed in stools. Studies of

the effect of changes in the growth environment of *C. difficile* have been performed in broth cultures. Toxin production by *C. difficile* in broth culture (peptone yeast broth) decreased when glucose or a mix of 9 amino acids was added to the medium (Karlsson, Burman et al. 1999). The most marked decrease in toxin production occurred with the addition of cysteine (or cysteine precursors) or proline to the broth culture (Karlsson, Lindberg et al. 2000). This would implicate nutrient deficiency as a stimulant of toxin production as well as conversion to the spore form. This suggests a common regulatory trigger that initiates sporulation while shutting down toxin production and secretion. The environment in the gut of patients with CDAD varies amongst patients with *C. difficile*. A previous study (Akerlund, Svenungsson et al. 2006) was unable to demonstrate a relationship between toxin present in the stools with *in vitro* toxin levels of *C. difficile* isolates from the stools.

Stool toxin levels within clonal isolates (as assessed by PFGE) of *C. difficile* showed some variation. This supports previously reported data that was unable to correlate to fecal toxin levels to PCR ribotypes (Akerlund, Svenungsson et al. 2006). The production of toxins by *C. difficile* is influenced by many different environmental factors. Nutrient deficiencies affect toxin production *in vitro* toxin levels in the stool (Karlsson, Burman et al. 1999; Karlsson, Lindberg et al. 2000). Differences in nutrients available in the gut could therefore have an impact on the toxin production by the infecting *C. difficile* strain. Also, the quantity of stool produced by patients with CDAD could affect toxin concentration present in the stool. Low volume of stool produced by a patient with CDAD could artificially increase the concentration of toxins in the stool even if the

infecting strain produced low levels of toxin. Our data showed some variability in toxin concentrations in stools despite the isolated strains belonging to the same PFGE types, for example toxin concentrations in stool for PFGE profile 0122 ranged from 1-3$\log_{10}$ cpeU/mL. Further the discriminatory power of current typing methods of *C. difficile* does not always allow significant distinction between isolates of *C. difficile* as is evidenced by the emergence of sub-types of PCR ribotype 001, the epidemic clone circulating in the United Kingdom (Fawley, Freeman et al. 2003; Fawley, Parnell et al. 2005; Northey, Gal et al. 2005). Information on the molecular mechanisms governing toxin production *in vitro* has been useful in describing models to explain the hyper toxin production by the NAP1 strain. However, these models need to be confirmed in a system that is able to mimic conditions *in vivo* such as the hamster model of disease (Bartlett, Chang et al. 1978) or the recently described human gut model (Freeman, O'Neill et al. 2003).

We were able to demonstrate that toxin production started earlier in the growth curve for the NAP1 strain compared to other clinical isolates. Toxin production in the NAP1 strain was first seen at 8-12 hours incubation in broth culture as compared to 16-24 hours for non-NAP1 strains. The NAP1 strain produced 2$\log_{10}$ higher concentrations of toxin B compared to non-NAP1 strains. Our results in the static broth culture confirm the findings of (Freeman, Baines et al. 2007) in the *in vitro* gut model who found that toxin is produced earlier in growth in the NAP1 strain. When comparing growth kinetics and toxin production of the NAP1 strain to the UK epidemic strain (PCR ribotype 001) in the gut model there was no difference in the maximum toxin levels produced by the two

strains (Freeman, Baines et al. 2007). The observation was made that toxin production in the NAP1 strain started during stationary phase as has been observed for other *C. difficile* isolates tested in the gut model. However, for the NAP1 strain *C. difficile* grew to the stationary phase earlier than the PCR ribotype 001 strain, explaining why toxin was detected earlier for the NAP1 isolate (Freeman, Baines et al. 2007). Further, the NAP1 strain produced toxin for 10 days longer than PCR ribotype 001 strain (23 versus 13 days). As the media was continually renewed in the *in vitro* gut model there was no accumulation of toxin in this system. This likely results in a maximum concentration of toxin that can accumulate as the system is continually renewed and secreted proteins are diluted in the fresh media. In the static culture model the earlier toxin production results is a greater accumulation of toxin. However, we were unable to determine if the accumulation of toxin B in broth culture was due to an increased rate of toxin production or by maintaining toxin production over time.

While not studied here, host factors are an important factor in acquisition and severity of CDAD. Patient factors such as healthy gut microflora, gastric acidity and normal peristalsis all have a role in preventing colonization by *C. difficile* (Kelly and LaMont 1998). A strong humoral immune response to toxin A has been correlated to asymptomatic carriage of *C. difficile* as measured by serum and fecal antibody levels (Kyne, Warny et al. 2000). Specifically, the presence of neutralizing antibodies to toxin A in the fecal matter or serum of patients prior to colonization with *C. difficile* was significantly linked to asymptomatic colonization. Further, a strong humoral immune response significantly decreased the risk of recurrence of CDAD (Aronsson, Granstrom

et al. 1985). Host immunity to *C. difficile* may affect parameters such as toxin and spore levels in the stools of patients with CDAD.

Our data demonstrated that the NAP1 strain of *C. difficile* had more efficient sporulation as compared to other non-NAP1 clinical isolates. Spores are a metabolically inactive form of *C. difficile* and as such are unable to produce toxin (Akerlund, Svenungsson et al. 2006). As previously reported (Ketley, Mitchell et al. 1986), the ATCC 43255 strain was inefficient at forming spores in broth culture with the maximum level of spores produced by 72 hours being only $1\log_{10}$ spores/mL produced. The NAP1 strain was far more efficient at forming spores. Spores were detected within 24 hours in the NAP1 strain with maximum spores of $\sim 7\log_{10}$ by 32 hours. *C. difficile* spores are key to the nosocomial transmission of *C. difficile* on the hands of caregivers or via shedding into the environment. Sporulation and toxin production are both stress response mechanisms in *C. difficile* (Akerlund, Svenungsson et al. 2006). Our data supports a previous study (Warny, Pepin et al. 2005) that found that the NAP1 strain produced higher spore levels in broth culture than clinical toxinotype 0 isolates when spores were enumerated by spore staining. This observation contradicts previous findings that efficient spore producers do not produce high levels of toxin (Akerlund, Svenungsson et al. 2006). However, spore staining provides a rather crude estimate of spore counts in culture and no results were shown to support Warny's claim that the NAP1 strain produced high levels of spores in broth culture. The NAP1 strain of *C. difficile* has been identified as the clonal strain of *C. difficile* in outbreaks of CDAD in Canada, the US, Belgium, France and the Netherlands (MacCannell, Louie et al. 2006; Tachon, Cattoen et al. 2006; van den Hof, van der Kooi

et al. 2006; Curry, Marsh et al. 2007). The ability of the NAP1 strain to convert efficiently from the vegetative to the spore form likely increases the rate of survival of the NAP1 strain in the environment, increasing the nosocomial transmission. The NAP1 strain has also been shown to produce high levels of biologically active toxin in broth culture. If *in vitro* findings indicate high levels of biologically active toxin and efficient spore formation for the NAP1 strain mimics CDAD in the gut of patients then this strain would be expected to cause severe disease and also be easily spread in the nosocomial environment.

The ability of *C. difficile* to persist and be transmitted is closely related to the dissemination of spores in the environment. *C. difficile* spores have been identified in the nosocomial environment on floors, walls, handrails, toilet, sinks etc. Also, *C. difficile* has been identified on the hands of healthcare workers and patients with CDAD (Fekety, Kim et al. 1981). We found that the NAP1 strain was able to efficiently convert from the vegetative to the spore form efficiently, early in the stationary phase with broth cultures predominantly in the spore form by 32 hours. This would facilitate the spread of *C. difficile* in the hospital environment. This is in contrast to the *C. difficile* ATCC 43255 strain which produces low levels of spores and high levels of toxin by 80 hours. The combined abilities of the NAP1 strain to produce high levels of toxin, causing more severe disease, and efficiently convert to the spore form, facilitating nosocomial spread, would increase the potential of the strain to cause an outbreak. However, there is evidence that NAP1-like strains are detected and they do not lead to outbreaks. Although intuitively environmental contamination by *C. difficile* spores would increase

transmission of *C. difficile* to the best of the authors knowledge no studies have been performed to date linking rates of environmental contamination to the incidence of CDAD.

Our data demonstrated variations in the efficiency of sporulation of *C. difficile* isolates in broth culture as well as in the stools of patients with CDAD. Sporulation as a mechanism of survival in unfavourable conditions has been described for all members of the *Bacillus* and *Clostridium* genera. Although sporulation has been described in depth for *Bacillus subtilis* and extended as a model for sporulation in *Clostridium* species, *C. difficile* is missing certain regulatory mechanisms that are necessary for efficient sporulation in the *B. subtilis* model. A phage-like insertional sequence $skin^{Cd}$ has recently been described in *C. difficile* (Haraldsen and Sonenshein 2003). The $skin^{Cd}$ inserts into and disrupts the transcription of the *sigK* gene, inhibiting the initiation of conversion to the spore form. Regulated excision of the $skin^{Cd}$ sequence from the *sigK* gene activates transcription of *sigK* controlling the initiating transcription of early sporulation genes in a regulated manner. $Skin^{Cd-}$ mutants are sporulation deficient supporting the essential role of this mechanism for sporulation in *C. difficile* isolates (Haraldsen and Sonenshein 2003). Differences in the $skin^{Cd}$ regulatory mechanism between *C. difficile* isolates may provide insight into the variations we observed in toxin production between the NAP1 strain and ATCC 43255. This work is currently in progress in this laboratory.

In our static broth culture system spores were detected in stationary phase when nutrients started to become depleted. During stationary phase toxin titres had reached maximum

levels, indicating that *C. difficile* was no longer secreting fresh toxins. The toxin that was detected in the culture supernatants during stationary phase was a result of residual toxin. A chemostatic gut model has been established to study *C. difficile* growth kinetics and toxin production. Briefly, this model consists of three vessels set up in series. The first vessel is seeded with *C. difficile* negative pooled fecal slurry then the series of vessels is continuously fed with fresh media. The fecal flora in this model is allowed to stabilize and the first vessel is inoculated with *C. difficile* spores. Germination of *C. difficile* spores is induced when the system is challenged with clindamycin, which knocks down the levels of normal flora and stimulates germination of the *C. difficile* spores (Freeman, Baines et al. 2007). In the gut model, proliferation of *C. difficile* in the vegetative form coincided with a concomitant increase in toxin A and B and a decrease in spore levels. In the continuously fed gut model as the normal gut flora was re-established after clindamycin was removed from the system, vegetative *C. difficile* was converted to spores and toxin secretion decreased (Freeman, Baines et al. 2007). As fresh media was fed into the system toxin concentrations in the three vessels decreased. While *C. difficile* spores are metabolically inactive and unable to produce toxin it is possible that a common trigger is responsible for shutting down toxin production and stimulating spore production. As such, both models support *C. difficile* being unable to produce toxin during spore conversion or while in the spore form.

Our data demonstrated that 12.5% of clinical isolates for 2000-2001 carried *cdtB*. This is slightly higher than the reported frequency of binary toxin in toxinogenic *C. difficile* (6-10%) (Popoff, Rubin et al. 1988; Stubbs, Rupnik et al. 2000; Geric, Johnson et al. 2003).

Despite carrying the *cdtB* gene these strains were not associated with the two outbreaks experienced over this time frame. Our data suggests that although binary toxin has been reported in the NAP1 outbreak strain it is not the sole virulence factor that facilitates outbreaks. The role of binary toxin in the pathogenesis of *C. difficile* has not been completely defined. However, the binary toxin genes (*cdtA/B*) have been amplified from non-toxinogenic isolates of *C. difficile* isolated from patients with antibiotic associated disease (Geric, Johnson et al. 2003). Hamsters infected with toxin A and toxin B negative, binary toxin positive isolates of *C. difficile* developed fluid accumulation in the ileal loop assay. However, purified binary toxin was unable to cause damage to the epithelial layer or lethality in the hamster model of CDAD (Geric, Carman et al. 2006).

The presence of the binary toxin locus has been identified in strains of *C. difficile* with variant PaLoc types. The putative truncation of the *tcdC* gene has been linked to the presence of binary toxin. Our data demonstrated that the binary toxin genes were present in *C. difficile* strains with the *tcdC* alleles, *tcdC-sc1* (NAP1-like) and *tcdC-sc13* (homologous to wild-type) but not in others. The presence of binary toxin has been associated with variations in the PaLoc, specifically the NAP1 strain *tcdC* truncation (Stare, Delmee et al. 2007). Our data confirmed that the *cdtA/B* gene (binary toxin) was associated with some *tcdC* alleles and not with others.

Toxin production and conversion to the spore form are both methods used by *C. difficile* to survive adverse conditions. As such it has been proposed that these two processes may share a common molecular regulatory mechanism. An early study (Kamiya, Ogura et al.

1992) showed that inhibition of sporulation in broth culture was accompanied by a reduction in maximal toxin production in culture. However other studies have rejected a link between toxin production and sporulation (Ketley, Mitchell et al. 1986). In our broth culture model spores were detected after vegetative *C. difficile* had reached stationary phase for all strains evaluated. However, there were variations in how soon and the rate at which strains converted from the vegetative to the spore form. The NAP1 strain showed rapid efficient sporulation. This was a novel finding for a hyper toxin producing isolate of *C. difficile*.

In summary, we were unable to conclusively identify all of the factors involved in the regulation of toxin production but our data does provide insight into the regulatory role of *tcdC*, *tcdR* and *tcdE* genes of the PaLoc genes. This study found that the NAP1 strain formed spores efficiently in broth culture, to the authors knowledge this is a novel finding for the NAP1 strain. This suggests that the NAP1 strain has an efficient mechanism to ensure it can survive in the nosocomial environment for extended periods of time. The binary toxin locus has been identified in the NAP1 strain and has been suggested as a factor that may facilitate outbreaks. However, our study was unable to detect binary toxin in the NAP1-unrelated outbreak strains from our facility. The data presented in our study supports the early and increased production of biologically active toxin A and B by the NAP1-like strain of *C. difficile* as compared to the non-NAP1-like strains. However, the results of our study do not support the current model of hyperproduction in the NAP1 strain which attributes hyper toxin production to the 18 bp and 1 bp mutations to TcdC, the putative negative regulator.

In the past decade work has been done to explain the virulence of *C. difficile*, despite this the factors that influence the ability of *C. difficile* to cause large epidemics are poorly understood. Studies that could help to further explain the transmission and pathogenesis of *C. difficile* are necessary (Loo, Poirier et al. 2005; Pepin, Valiquette et al. 2005). To best of the author's knowledge there have been no studies that have prospectively followed patients with CDAD to determine toxin and spore levels in the stools of patients as disease progresses and during treatment. This could provide useful information on the persistence of *C. difficile* in the environment as well how toxin levels in stool are affected by treatment. One of the areas of *C. difficile* pathogenesis that is poorly understood is the conversion of *C. difficile* to the spore form. While a model of sporulation has been described in *B. subtilis*, *C. difficile* does not appear to use the same mechanism (Hoch 1993). A recent study (Haraldsen and Sonenshein 2003) identified an insertion sequence ($skin^{Cd}$) that inserts into *sigK* and is necessary for efficient sporulation in *C.difficile*. Further studies are need to define the role of the $skin^{Cd}$ insertion in spore conversion.

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